04-23

JC17 Rec'd PCT/PTO 20 APR 2001

Form PTO-1390 (REV 11-98)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 789-47
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		US. APPLICATION NO. (If known, see 37 CFR 1.5) 09/830146
INTERNATIONAL APPLICATION NO. PCT/CA99/00987	INTERNATIONAL FILING DATE 21 October 1999	PRIORITY DATE CLAIMED 21 October 1998

TITLE OF INVENTION
METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

APPLICANT(S) FOR DO/EO/US
BEAUDOIN, Adrien; MARTIN, Geneviève

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [x] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. [x] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. [x] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [x] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [x] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. [x] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) [unsigned].

10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [x] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.

14. [x] A substitute specification.

15. [ ] A change of power of attorney and/or address letter.

16. [ ] Other items or information:

EXPRESS MAIL LABEL NO. EL 740156190 US

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/830146	INTERNATIONAL APPLICATION NO. PCT/CA99/00987	ATTORNEY'S DOCKET NUMBER 789-47

17. [X] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**

CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	44 -20 =	14	X **$18.00**	$252.00
Independent claims	5 -3 =	2	X **$80.00**	$160.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$
TOTAL OF ABOVE CALCULATIONS =				$1272.00
Reduction by 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				$636.00
SUBTOTAL =				$636.00
Processing fee of **$130.00** for furnishing the English translation later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.429(f)). +				$
TOTAL NATIONAL FEE =				$636.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$
TOTAL FEES ENCLOSED =				$636.00
			Amount to be: refunded	$
			Charged	$

a. [X] A check in the amount of $ 636.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. 50-0951 in the amount of $ 0.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0951. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Gregory A. Nelson
Akerman, Senterfitt & Eidson, P.A.
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SIGNATURE

Joseph W. Bain
NAME

34,290
REGISTRATION NUMBER

P1011358;1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: BEAUDOIN et al

Application No. Examiner:

Filed: Herewith Group Art Unit:

For: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

PRELIMINARY AMENDMENT

Box Patent Applications
Commissioner for Patents
Washington, DC 20231

Sir:

Prior to examination on the merits, please amend the above-identified application as follows:

IN THE SPECIFICATION

Pages 29 and 30 were unintentionally omitted from the response to the Written Opinion during International Preliminary Examination. Please therefore insert the following text after table 15 and before the claims:

- - TABLE 16. TOCOPHEROL, ALL-*trans* RETINOL AND CHOLECALCIFEROL CONTENT IN KRILL OIL (*E. pacifica*)

alpha-tocopherol by HPLC (IU)	
Fraction I [a)]	0,91
Fraction II [b)]	0,83

gamma-tocopherol by HPLC µg/g	
Fraction I [a)]	Tr
Fraction II [b)]	Tr
delta-tocopherol by HPLC µg/g	
Fraction I [a)]	N.D.
Fraction II [b)]	N.D.
all-*trans* retinol by HPLC (IU)	
Fraction I [a)]	395,57
Fraction II [b)]	440,47
cholecalciferol by HPLC (IU)	
Fraction I [a)]	N.D.
Fraction II [b)]	N.D.

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology,

Halifax, Nova Scotia.

Data expressed per gram of krill oil.

[a)] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.

[b)] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

TR = trace

N.D. = not detected

Conversion : Vitamin	alpha-tocopherol	mg/g oil x 1,36 = International Unit
	All-*trans* retinol	µg/g ÷ 0,3 = International Unit

TABLE 17. ASTAXANTHIN AND CANTHAXANTHIN CONTENT OF KRILL OIL (*E. pacifica*)

Asthaxantin (µg/g oil)	
Fraction I [a)]	93,1
Fraction II [b)]	121,7
Canthaxanthin (µg/g oil)	
Fraction I [a)]	270,4
Fraction II [b)]	733,0

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology,
Halifax, Nova Scotia.

[a)] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.

[b)] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

TABLE 18. OPTIMAL CONDITIONS FOR LIPID EXTRACTION OF AQUATIC ANIMAL TISSUES (suggested procedure)

STEP	CONDITIONS
Grinding (if particles > 5mm)	4°C
Lipid extraction	sample-acetone ratio of 1:6 (w/v) 2h (including swirling 20 min) 4°C
Filtration	organic solvent resistant filter

	under reduced pressure
Washing	sample-acetone ratio of 1:2 (w/v) pure and cold acetone
Filtration	organic solvent resistant filter under reduced pressure
Evaporation	under reduced pressure
Oil-water separation	4°C
Lipid extraction	sample: ethyl acetate ratio of 1:2 (w/v)[a)] pure ethyl acetate 30 min 4°C [b)]
Filtration	organic solvent resistant filter under reduced pressure
Evaporation	under reduced pressure

[a)]: Ethanol can be replaced by isopropanol, *t*-butanol or ethyl acetate.

[b)]: 25 °C when using *t*-butanol.

TABLE 19: PROTEOLYTIC ACTIVITY OF KRILL RESIDU USING LACTOSERUM AS THE SUBSTRATE, AT 37 °C, PH 7.0 FOR A RATIO ENZYME:SUBSTRATE OF 1:43

Time (min)	Amino acids released (µmoles)	Enzymatic rate (µmoles/min)	Specific activity	enzymatic

			(μmoles/min/mg*)
15	28.76	1.917	0.164
30	43.74	0.999	0.125
170	98.51	0.322	0.050
255	177.26	0.308	0.060

* total quantity of enzymes in hydrolysis media - -

IN THE CLAIMS:

1. (Amended) A method for extracting lipid fractions from marine and aquatic animal material, said method comprising the steps of:

(a) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents;

(c) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(d) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(e) separating the liquid and solid contents;

(f) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(g) recovering the solid contents.

4. (Amended) A method as in claim 1, wherein steps (b) and (d) are conducted under inert gas atmosphere.

5. (Amended) A method as in claim 1, wherein steps (b) and (e) are effected by techniques selected from filtration, centrifugation and sedimentation.

6. (Amended) A method as in claim 1 , wherein steps (c) and (f) are effected by techniques selected from vacuum evaporation, flash evaporation and spray drying.

7. (Amended) A method as in claim 1, wherein after step (b) and before step (c), the method additionally comprises the intervening step of washing the solid contents with the solvent and adding the resulting washing solution to the liquid contents of step (b).

8. (Amended) A method as in claim 1, wherein after step (e) and before step (f), the method additionally comprises the intervening step of washing the solid contents with the organic solvent selected in step (d).

9. (Amended) A method as in claim 1, wherein prior to step (a) the marine and aquatic animal material is finely divided.

10. (Amended) A method as in claim 1, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

11. (Amended) A method as in claim 1, wherein said marine and aquatic animal is zooplancton.

12. (Amended) A method as in claim 11, wherein said zooplancton is selected from krill and *Calanus*.

Please cancel claim 13 without prejudice.

14. (Amended) A method as in claim 1, wherein said marine and aquatic animal is fish filleting by-products.

15. (Amended) A method for extracting an astaxanthin-and-canthaxantin-containing lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, said method comprising the steps of:

(a) placing said animal material in a ketone solvent to achieve an extraction of the soluble lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents;

(c) recovering a lipid-rich fraction from the liquid contents by evaporation of the solvent present in the liquid contents;

whereby an astaxanthin-and-canthaxantin-containing lipid fraction is obtained.

16.(Amended) A method for extracting a lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, said method comprising the steps of:

(a) placing said animal material in a solvent mixture comprising acetone and ethanol to achieve an extraction of the soluble lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents;

(c) recovering a lipid-rich fraction from the liquid contents by evaporation of the solvents present in the liquid contents;

whereby a lipid fraction is obtained.

17. (Amended) A method as in claim 15, wherein the animal material is selected from krill and *Calanus*.

Please cancel claim 18 without prejudice

19. (Amended) A method as in claim 15, wherein during step (a), the animal material is homogenized.

20. (Amended) A method as in claim 15, wherein steps (b) and (d) are conducted under inert gas atmosphere.

21. (Amended) A method as in claim 15, wherein step (b) is effected by a technique selected from filtration, centrifugation and sedimentation.

22. (Amended) A method as in claim 15, wherein step (c) is effected by a technique selected from vacuum evaporation, flash evaporation and spray drying.

23. (Amended) A method as in claim 15, wherein after step (b) and before step (c), the method additionally comprises a step of washing said solid contents with solvent and adding the resulting washing solution to the liquid contents of step (b).

24. (Amended) A method as in claim15, wherein prior to step (a) the marine and aquatic animal material is finely divided.

25. (Amended) A method as in claim 15, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

26. (Amended) A krill lipid extract characterized in that the carotenoid content in asthaxanthin is at least about 75 mg/g of krill extract, and the carotenoid content in canthaxanthin is at least about 250 mg/g of krill extract.

27. (Amended) A method of lipid extraction as in claim 1, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

Please cancel claims 28 and 29 without prejudice

30. (Amended) A method of lipid extraction as in claim 15, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

31. (Amended) A method for extracting lipid fractions from marine and aquatic animal material, said method comprising the steps of:

(a) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;
(b) separating the liquid and solid contents;
(c) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;
(d) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;
(e) separating the liquid and solid contents;
(f) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

whereby lipid fractions are obtained.

32. (Amended) A method of lipid extraction as in claim 31, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

33. (Amended) A lipid fraction extracted from marine and aquatic animal material, by a method comprising the steps of:

(g) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(h) separating the liquid and solid contents;

(i) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(j) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(k) separating the liquid and solid contents;

(l) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(m) recovering the solid contents.

Please cancel claims 34 and 35 without prejudice.

36. (New) A method as in claim 1, wherein the ketone solvent is acetone.

37. (New) A method as in claim 1, wherein the alcohol is selected from the group of ethanol, isopropanol and *t*-butanol.

38. (New) A method as in claim 1, wherein the ester of acetic acid is ethyl acetate.

39. (New) A method as in claim 9, wherein the marine and aquatic animal material is finely divided to an average particle size of not more than 5mm.

40. (New) A method as in claim 15, wherein said marine and aquatic animal material is viscera.

41. (New) A method as in claim 15, wherein the ketone solvent is acetone.

42. (New) A method as in claim 16, wherein said marine and aquatic animal material is viscera.

43. (New) A method as in claim 16, wherein the animal material is selected from krill and *Calanus.*

44. (New) A method as in claim 24, wherein the animal material is finely divided to an average particle size of not more than 5mm.

45. (New) A krill lipid extract as in claim 26, wherein the carotenoid content in asthaxanthin is at least about 90 mg/g of krill extract.

46. (New) A krill lipid extract as in claim 26, wherein the carotenoid content in canthaxanthin is at least about 270 mg/g of krill extract.

47. (New) A method as in claim 1, wherein the solid contents of step (e) is recovered and consists of a dehydrated residue containing active enzymes.

48. (New) A method as in claim 31, wherein the ketone solvent is acetone.

49. (New) A method as in claim 31, wherein the alcohol is selected from the group of , ethanol, isopropanol and *t*-butanol.

50. (New) A method as in claim 31, wherein the ester of acetic acid is ethyl acetate.

51. (New) A method of lipid extraction as in claim 31, wherein the solid contents of step (e) is recovered and consists of a dehydrated residue containing active enzymes.

REMARKS

A complete copy of the specification which is to form the basis for the US National Phase of PCT/CA99/00987 is submitted herewith.

Respectfully submitted

Date: 4/20/01

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Docket No. 789-47

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: BEAUDOIN et al

Application No. Examiner:

Filed: Herewith Group Art Unit:

For: METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

ATTACHMENT TO PRELIMINARY AMENDMENT SHOWING MODIFICATIONS

Box Patent Applications
Commissioner for Patents
Washington, DC 20231

Sir:

In accordance with 37 CFR §1.121, the modifications made to the specification and claims are as follows:

IN THE SPECIFICATION

After table 15 and before the claims (modifications indicated with respect to the PCT application as originally filed)

TABLE [17]16. TOCOPHEROL, ALL-*trans* RETINOL AND CHOLECALCIFEROL CONTENT IN KRILL OIL (*E. pacifica*)

alpha-tocopherol by HPLC (IU)	
Fraction I [a)]	0,91
Fraction II [b)]	0,83

gamma-tocopherol by HPLC µg/g	
Fraction I [a)]	Tr
Fraction II [b)]	Tr
delta-tocopherol by HPLC µg/g	
Fraction I [a)]	N.D.
Fraction II [b)]	N.D.
all-*trans* retinol by HPLC (IU)	
Fraction I [a)]	395,57
Fraction II [b)]	440,47
cholecalciferol by HPLC (IU)	
Fraction I [a)]	N.D.
Fraction II [b)]	N.D.

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology,

Halifax, Nova Scotia.

Data expressed per gram of krill oil.

[a)] : Extraction made with a sample-acetone ratio of 1·6 (w/v), incubated 2h at 4°C.

[b)] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

TR = trace

N.D. = not detected

Conversion : Vitamin	alpha-tocopherol	mg/g oil x 1,36 = International Unit
	All-*trans* retinol	µg/g ÷ 0,3 = International Unit

TABLE [18]17. ASTAXANTHIN AND CANTHAXANTHIN CONTENT OF KRILL OIL (*E. pacifica*)

Asthaxantin (µg/g oil)	
Fraction I [a)]	93,1
Fraction II [b)]	121,7
Canthaxanthin (µg/g oil)	
Fraction I [a)]	270,4
Fraction II [b)]	733,0

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology,
Halifax, Nova Scotia.

[a)] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.

[b)] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

TABLE [19]18. OPTIMAL CONDITIONS FOR LIPID EXTRACTION OF AQUATIC ANIMAL TISSUES (suggested procedure)

STEP	CONDITIONS
Grinding (if particles > 5mm)	4°C
Lipid extraction	sample-acetone ratio of 1:6 (w/v) 2h (including swirling 20 min) 4°C
Filtration	organic solvent resistant filter

	under reduced pressure
Washing	sample-acetone ratio of 1:2 (w/v) pure and cold acetone
Filtration	organic solvent resistant filter under reduced pressure
Evaporation	under reduced pressure
Oil-water separation	4°C
Lipid extraction	sample: ethyl acetate ratio of 1:2 (w/v)[a)] pure ethyl acetate 30 min 4°C [b)]
Filtration	organic solvent resistant filter under reduced pressure
Evaporation	under reduced pressure

[a)]: Ethanol can be replaced by isopropanol, *t*-butanol or ethyl acetate.

[b)]: 25 °C when using *t*-butanol.

TABLE [20]19: PROTEOLYTIC ACTIVITY OF KRILL RESIDU USING LACTOSERUM AS THE SUBSTRATE, AT 37 °C, PH 7.0 FOR A RATIO ENZYME:SUBSTRATE OF 1:43

Time (min)	Amino acids released (µmoles)	Enzymatic rate (µmoles/min)	Specific activity	enzymatic

			(μmoles/min/mg*)
15	28.76	1.917	0.164
30	43.74	0.999	0.125
170	98.51	0.322	0.050
255	177.26	0.308	0.060

* total quantity of enzymes in hydrolysis media

IN THE CLAIMS

Modifications indicated with respect to the claims existing after International Preliminary Examination

1. (Amended) A method for extracting lipid fractions from marine and aquatic animal material, said method comprising the steps of:

(n) placing marine and aquatic animal material in a ketone solvent[, preferably acetone] to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(o) separating the liquid and solid contents;

(p) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(q) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol[, preferably ethanol, isopropanol or *t*-butanol] and esters of acetic acid[, preferably ethyl acetate] to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(r) separating the liquid and solid contents;

(s) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(t) recovering the solid contents.

4. (Amended) A method as in [any of] claim[s] 1 [to 3], wherein steps (b) and (d) are conducted under inert gas atmosphere.

5. (Amended) A method as in [any of] claim[s] 1 [to 4], wherein steps (b) and (e) are effected by techniques selected from filtration, centrifugation and sedimentation.

6. (Amended) A method as in [any of] claim[s] 1 [to 5] , wherein steps (c) and (f) are effected by techniques selected from vacuum evaporation, flash evaporation and spray drying.

7. (Amended) A method as in [any of] claim[s] 1 [to 6], wherein after step (b) and before step (c), the method additionally comprises the intervening step of washing the solid contents with the solvent and adding the resulting washing solution to the liquid contents of step (b).

8. (Amended) A method as in [any of] claim[s] 1 [to 7], wherein after step (e) and before step (f), the method additionally comprises the intervening step of washing the solid contents with the organic solvent selected in step (d).

9. (Amended) A method as in [any of] claim[s] 1 [to 8], wherein prior to step (a) the marine and aquatic animal material is finely divided[, preferably to an average particle size of 5mm or less].

10. (Amended) A method as in claim[s] 1 [to 9], wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C [or less].

11. (Amended) A method as in claim[s] 1 [to 10], wherein said marine and aquatic animal is zooplancton.

12. (Amended) A method as in claim 11, wherein said zooplancton is selected from krill and *Calanus*.

14. (Amended) A method as in claim[s] 1 [to 10], wherein said marine and aquatic animal is fish filleting by-products.

15. (Amended) A method for extracting an astaxanthin-and-canthaxantin-containing lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, [preferably viscera,] said method comprising the steps of:

(a) placing said animal material in a ketone solvent[, preferably acetone] to achieve an extraction of the soluble lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents;

(c) recovering a lipid-rich fraction from the liquid contents by evaporation of the solvent present in the liquid contents;

whereby an astaxanthin-and-canthaxantin-containing lipid fraction is obtained.

16.(Amended) A method for extracting a lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, [preferably viscera,] said method comprising the steps of:

(a) placing said animal material in a solvent mixture comprising acetone and ethanol to achieve an extraction of the soluble lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents;

(c) recovering a lipid-rich fraction from the liquid contents by evaporation of the solvents present in the liquid contents;

whereby a lipid fraction is obtained.

17. (Amended) A method as in claim 15 [or 16], wherein the animal material is selected from krill and *Calanus*.

19. (Amended) A method as in [any one of] claim[s] 15 [to18], wherein during step (a), the animal material is homogenized.

20. (Amended) A method as in [any one of] claim[s] 15 [to 19], wherein steps (b) and (d) are conducted under inert gas atmosphere.

21. (Amended) A method as in [any one of] claim[s] 15 [to 20], wherein step (b) is effected by a technique selected from filtration, centrifugation and sedimentation.

22. (Amended) A method as in [any one of] claim[s] 15 [to21], wherein step (c) is effected by a technique selected from vacuum evaporation, flash evaporation and spray drying.

23. (Amended) A method as in [any one of] claim[s] 15 [to 22], wherein after step (b) and before step (c), the method additionally comprises a step of washing said solid contents with solvent and adding the resulting washing solution to the liquid contents of step (b).

24. (Amended) A method as in [any one of] claim[s] 15 [to 23], wherein prior to step (a) the marine and aquatic animal material is finely divided[, preferably to an average particle size of 5mm or less].

25. (Amended) A method as in [any one of] claim[s] 15 [to 23], wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C [or less].

26. (Amended) A krill lipid extract characterized in that the carotenoid content in asthaxanthin is at least about 75 [and preferably at least about 90] mg/g of krill extract, and the carotenoid content in canthaxanthin is at least about 250 mg/g [and preferably at least about 270 mg/g] of krill extract.

27. (Amended) A method of lipid extraction as in [any one of] claim[s] 1 [to 14], wherein the solid contents of step (b) [and/or e)] is recovered and consists of a dehydrated residue containing active enzymes.

30. (Amended) A method of lipid extraction as in [any one of] claim[s] 15 [to 25], wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

31. (Amended) A method for extracting lipid fractions from marine and aquatic animal material, said method comprising the steps of:

(a) placing marine and aquatic animal material in a ketone solvent[, preferably acetone] to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents;

(c) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(d) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol[, preferably ethanol, isopropanol or *t*-butanol] and esters of acetic acid[, preferably ethyl acetate] to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(e) separating the liquid and solid contents;

(f) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

whereby lipid fractions are obtained.

32. (Amended) A method of lipid extraction as in claim 31, wherein the solid contents of step (b) [and/or e)] is recovered and consists of a dehydrated residue containing active enzymes.

33. (Amended) [The] A lipid fraction [obtained by the method of any one of claims 1 to 25, 27, and 30 to 32] extracted from marine and aquatic animal material, by a method comprising the steps of:

(g) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(h) separating the liquid and solid contents;

(i) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(j) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(k) separating the liquid and solid contents;

(l) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(m) recovering the solid contents.

Respectfully submitted

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TITLE OF THE INVENTION

METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

FIELD OF THE INVENTION

This invention relates to the extraction of lipid fractions from marine and aquatic animals such as krill, *Calanus*, fish and sea mammals. More specifically, this invention relates to an improved method of extracting lipid fractions by dehydration with solvents and recovering a solid residue rich in active enzymes.

BACKGROUND OF THE INVENTION

Lipid fractions obtained from marine and aquatic animals such as krill, *Calanus*, fish and sea mammals have various applications:

Medical applications

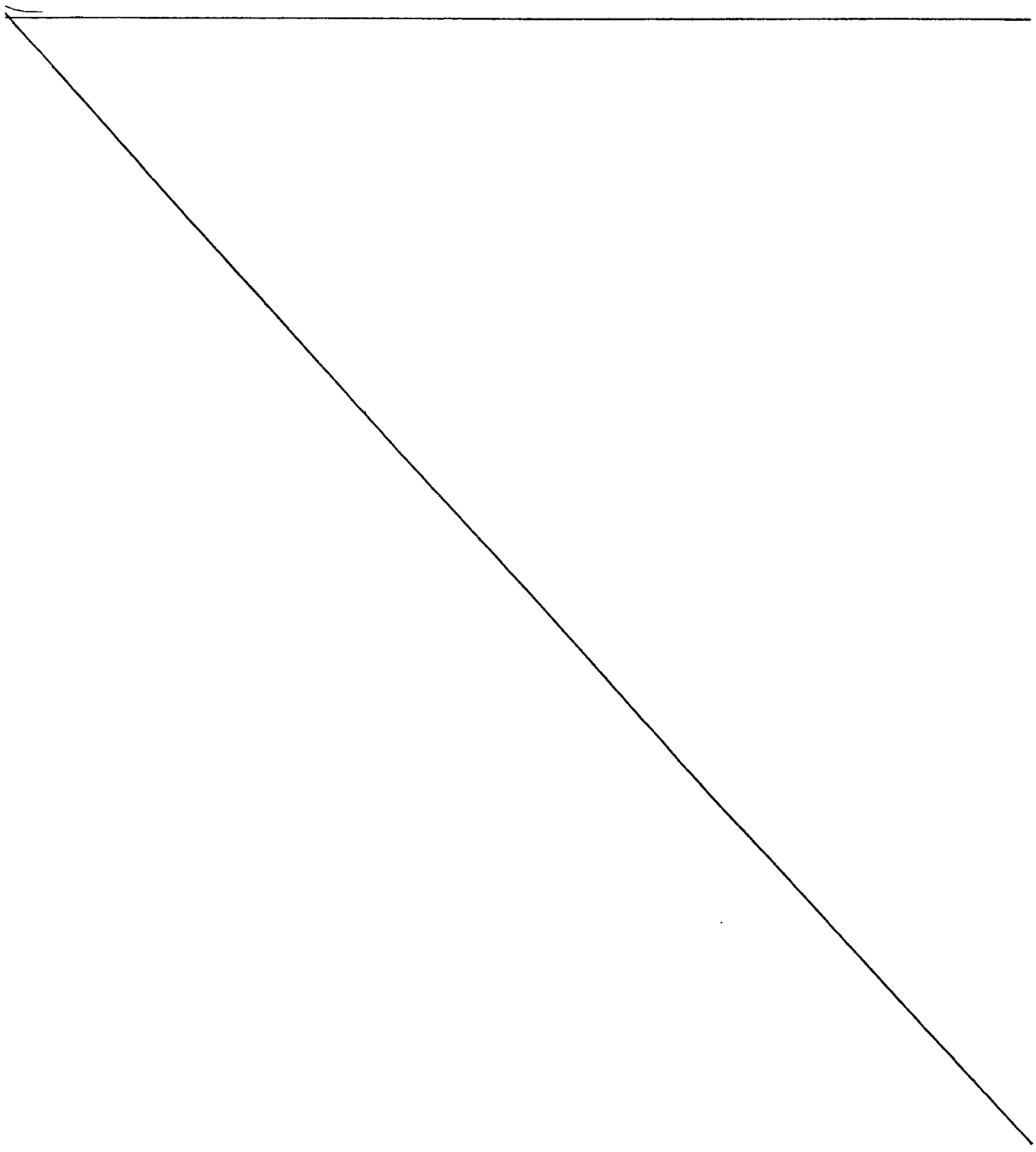
Marine and aquatic animal oils and fractions thereof contain various therapeutic agents. For example, it is reported that various marine and aquatic animal oils have anti-inflammatory properties. Marine and aquatic animal oils are also reported as helpful in reducing the incidence of cardiovascular disease. Also, some marine and aquatic animal oils are reported as suppressing the development of certain forms of lupus and renal diseases. As a further example, krill may be used as a source of enzymes for debridement of ulcers and wounds or to facilitate food digestion. Also marine and aquatic oils contain various antioxidants, which may have potential therapeutic properties.

Nutraceuticals

Considering the beneficial effects of omega-3 fatty acids, oils from krill, *Calanus* and fish could be used as dietary supplements to human diet. These fatty acids are essential for proper development of the brain and the eye. Marine and aquatic animal oils are also rich in liposoluble vitamins A, D and E and carotenoids.

Cosmetics

Various marine and aquatic animal oils are used for the production of moisturizing creams.



Fish farming

Among the lipids found in krill, *Calanus* and fish, high concentrations of fatty acids 20:5 (eicosapentaenoic acid) and 22:6 (docosahexaenoic acid) are present. These fatty acids are essential nutrients and are beneficial as fish feed. Furthermore, these essential nutrients are carried over in human diet by eating the fish grown on such diets.

Animal feed

Animal feed diets rich in omega-3 fatty acids may increase the level of unsaturated fatty acids and decrease cholesterol levels of meat. This property is already exploited in the poultry industry to improve the quality of eggs.

Various methods for extracting marine and aquatic animal oils are known. For example, it is known to extract fish oil using organic solvents such as hexane and ethanol. It is also known to measure the fat content in fish muscle tissue using solvents such as acetone.

USP 4,331,695 describes a method using pressurized solvents which are gaseous at room temperature, such as propane, butane or hexane. The extraction is performed at preferred temperatures of 15 to 80°C on shredded vegetable or finely divided animal products. The extracted oils are then made to precipitate under high pressure and elevated temperatures of 50 to 200°C. However, hexane is a poor extraction solvent for marine animals such as krill. Furthermore, the high temperatures used in the precipitation step negatively alters the lipids.

Canadian Patent Application 2,115,571 describes a method for extracting oils from various brown and read algae species. The method provides for example Soxhlet extraction using nearly pure ethanol for 40 hours.

USP 5,006,281 describes a method for extracting oil from marine and aquatic animals such as fish. The marine and aquatic animal is first treated with an antioxidant compound, finely divided and centrifuged to separate the oil phase from

the aqueous phase and solid phase. The oil phase is then further treated with antioxidant to remove undesirable odour or taste.

Canadian Patent 1,098,900 describes a method for extracting oils from krill. The method involves emulsifying fresh or defrosted krill in an aqueous medium. The oil fraction is recovered by centrifugation.

Folch in the article published in the year 1957 in J. biol. Chem. 226: 497-509 *"A simple method for the isolation and purification of total lipids from animal tissues"* proposes an extraction method using chloroform and methanol. This method is not commercially feasible because of the toxicity of the solvents involved.

However, prior art processes are generally commercially unfeasible or provide low quantitative yields. Thus, it is an object of the present invention to provide an improved marine and aquatic animal oil extraction method allowing recovery of a valuable lipid fraction and separate recovery of a valuable protein rich solid residue that comprises active enzymes.

Other objects and further scope of applicability of the present invention will become apparent from the detailed description given hereinafter. It should be understood, however, that this detailed description, while indicating preferred embodiments of the invention, is given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Gas-liquid chromatography of fatty acids from dry krill (chloroform-methanol)

Figure 2. Gas-liquid chromatography of fatty acids from dry krill (acetone)

Figure 3. Gas-liquid chromatography of fatty acids from frozen krill (acetone)

Figure 4. Gas-liquid chromatography of fatty acids from frozen krill (ethanol)

Figure 5. Gas-liquid chromatography of fatty acids from frozen krill (*t*-butanol)

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Before describing the present invention in detail, it is to be understood that the invention is not limited in its application to the process details described herein. The invention is capable of other embodiments and of being practised in various ways. It is also to be understood that the phraseology or terminology used herein is for the purpose of description and not limitation.

The method of the invention comprises suspending freshly collected marine and aquatic material in acetone. Lipids are extracted with a ketone such as acetone. This allows a rapid dehydration of animal tissue and a migration of the lipid fraction to the solvent. The dry residue is a valuable product rich in active enzymes.

In a preferred embodiment, the extraction is carried out by successive acetone and alcohol treatments. Preferred alcohols are isopropanol, and *t*-butanol. The alcohol may also be substituted with an ester of acetic acid such as ethyl acetate. The procedure produces two successive lipid fractions and a dry residue enriched in protein, including active enzymes. Recovery of total lipids is comparable to the Folch et al. (1957) procedure reported in the background of the invention. It has been tested with krill, *Calanus*, fish and shark tissues.

Surprisingly, it was found that successive extraction treatments as proposed by the present invention has a better yield in lipid extraction that single solvent system extractions. The extraction using two successive solvents which starts with a ketone such as acetone is especially advantageous since the acetone, in effect, dehydrates the animal tissue. Having the animal tissue in dehydrated form greatly facilitates the extraction process with the second solvent, alcohol or an ester of acetic acid such as ethyl acetate.

In the case of zooplancton such as krill and *Calanus* and in the case of fish-filleting by-products such as fish viscera, it is noted that extraction with acetone alone may be sufficient to allow a cost-effective recovery of lipid fractions and separate recovery of a dry solid product rich in proteins including active enzymes.

The general extraction method of the present invention will now be described. The starting material consisting of freshly harvested and preferably finely divided marine and aquatic animal material is subjected to acetone extraction, for at about two hours and preferably overnight. However extraction time is not critical to the yield of lipid extraction. To facilitate extraction, it is preferable to use particles of less than 5mm in diameter. Extraction is preferably conducted under inert atmosphere and at a temperature in the order of about 5°C or less.

Preferably, the beginning of the extraction will be conducted under agitation for about 10 to 40 minutes, preferably 20 minutes. Although extraction time is not critical, it

was found that a 2 hour extraction with 6:1 volume ratio of acetone to marine and aquatic animal material is best.

The solubilized lipid fractions are separated from the solid material by standard techniques including, for example, filtration, centrifugation or sedimentation. Filtration is preferably used.

After separation by filtration on an organic solvent resistant filter (metal, glass or paper) the residue is optionally washed with pure acetone, preferably two volumes (original volume of material) to recover yet more lipids. The combined filtrates are evaporated under reduced pressure. Optionally, flash evaporation or spray drying may be used. The water residue obtained after evaporation is allowed to separate from the oil phase (fraction I) at low temperature.

The solid residue collected on the filter is suspended and extracted with alcohol, such as ethanol, isopropanol, *t*-butanol or alternatively with ethyl acetate, preferably two volumes (original volume of material). The filtrate is evaporated leaving a second fraction of lipids (identified as fraction II). Although the extraction period is not critical, it was found that an extraction time of about 30 minutes is sufficient at temperatures below about 5°C.

Temperature of the organic solvents, except *t*-butanol, and temperature of the sample are not critical parameters, but it is preferable to be as cold as possible. However, in the case of *t*-butanol which is solid at room temperature, it is important to warm it before using it and to perform the extraction at 25 °C immediately.

<u>Comparative examples</u>

To compare the efficiency of the extraction process, a classical technique (Folch et al. 1957) using chloroform and methanol was applied to krill. This method is the reference for measuring efficiency of the extraction process. Another comparison has been made with a technique using hexane as the extraction solvent. Lipid recovery

by suspending lipid fractions in small volumes of their original solvents and measuring by gravimetry small aliquots after evaporation.

For all examples provided herein, the method of the present invention involving acetone extraction followed by extraction with a second solvent (ethyl acetate, for example) gave a translucent oil having appearance and properties more attractive than any oil obtained by the classical technique of Folch et al. (1957).

To analyze lipid composition, 780 µg of each extract was loaded on silica-gel plates and fractionated by thin layer chromatography, TLC (Bowyer et al. 1962) with the following solvents. Neutral lipids: hexane, ethyl ether, acetic acid (90:10:1, v/v) and phospholipids: chloroform, methanol, water (80:25:2, v/v). Fatty acid composition of *E. pacifica* was analyzed by gas liquid chromatography, GLC (Bowyer et al. 1962) including some modifications to the original technique: 2h at 65°C instead of 1h at 80°C, three washes with hexane instead of two and no wash with water.

To get rid of traces of organic solvents, lipid fractions I and II are warmed to about 125°C for about 15 minutes under inert atmosphere.

Fat was analyzed according to the American Oil Chemist's Society (AOCS). The following criteria have been used to analyze the lipids extracted: saponification and Wijs iodine indexes and moisture-volatile matter levels. Cholesterol content has also been determined by the method of Plummer 1987. The same analyzes and others have been made by an independent laboratory under Professor Robert Ackman's supervision (Canadian Institute of Fisheries Technology, DalTech, Dalhousie University, Halifax, Nova Scotia, Canada). This includes Wijs iodine index, peroxide and anisidine values, lipid class composition, fatty acid composition, free fatty acid FAME, cholesterol, tocopherol, all-*trans* retinol, cholecalciferol, astaxanthin and canthaxantin contents.

Table 1 shows that higher levels of lipids are extracted from dry krill by acetone followed by ethanol as compared to the classical procedure of Folch et al. (1957).

Table 2 shows the results of lipid extraction from frozen *Euphausia pacifica*, a species of krill from Pacific Ocean. Assuming an eighty percent content of water, the lipid content is comparable to dry krill as shown in Table 1. Isopropanol, *t*-butanol and ethyl acetate, as solvent for the second extraction, give a yield less important than ethanol, but are not necessarily less effective in lipid recovery since ethanol carries more impurities than isopropanol, *t*-butanol or ethyl acetate. Then, they can be used as second solvent after acetone as well. Variations between results from acetone extractions are mainly due to the water-oil separations. These separations are influenced by the quantity of residual acetone in the water-oil solution after acetone evaporation. This quantity of acetone varies from an experiment to another, because the evaporation system used at a small scale is less reproducible (at the industrial scale, the evaporation step will be optimized). Single solvents have also been tested to extract the totality of lipids from krill. This shows that ethyl acetate (1,37% extraction rate), as hexane (0,23% extraction rate) are not good solvents, compared to acetone alone (1,86% extraction rate, and even greater extraction rates with an efficient acetone evaporation system).

One of the main advantages of the procedure is the removal of bacteria from extracts (lipid fraction and solid protein-rich material). Indeed, samples of *E. pacifica* incubated in different ratios of acetone at 4°C for 112 days have been inoculated on NA medium containing Bacto™ beef extract 0,3%, Bacto™ peptone 0,5% and Bacto™ agar 1,5% (Difco Laboratories, Detroit, USA) then incubated at room temperature or 4°C for 18 days. No significant bacterial growth was observed at a ratio of 1 volume of acetone per gram of krill. At higher proportions of acetone (2 volumes and 5 volumes), there was no bacterial growth at all, which means that acetone preserves krill samples. Acetone is known as an efficient bactericidal and viricidal agent (Goodman et al. 1980).

Table 3 shows the yield of lipids from *M. norvegica*. The percentage of lipids (3,67%) is comparable to the one obtained with *E. pacifica* (3,11%) shown in Table 2. Variations can be attributable to diet and time (season) of collection, which are different for those two species.

Table 4 shows the influence of grinding on the efficiency of extraction of *M. norvegica* lipids. These extractions were carried out under optimal conditions and show the definite advantage of the procedure over the classical method (4,46 % versus 3,30 %). It also shows that grinding may be an important factor when the species is large (4,46% versus 3,53 %).

Table 5 reports on lipid extraction from *Calanus*. Considerable quantities of lipids were obtained. Some variations in *Calanus* species composition may explain the variations between experiments 1 and 2 (8,22 % and 10,90 % of fresh weight).

Tables 6-8 report the total amount of lipids extracted from fish tissue. The method of the present invention was demonstrated on mackerel, trout and herring. The method was demonstrated on peripheral tissues (mainly muscles) and viscera. Advantageously, the present method would permit the recovery of valuable lipid fractions from parts of fish that are usually wasted after the withdrawal of fillets of the fish. Those fish tissues not used after the transformation of the fish for human consumption could be stored in acetone, and lipids extracted therefrom in accordance with the present invention even if the method Folch [1957] recovers more lipid than our method. Indeed small amounts of lipids from mackerel (0.52% from viscera and 1,45% from tissues) have been extracted by the method of Folch after a first extraction with acetone and ethanol as described in the present invention. Comparative extractions with the method described in the present invention carried out in parallel with the method of Folch on trout and herring show superior recovery with the latter. However, it is noteworthy that the Folch method can not be applied for the recovery of lipids for commercial uses (because of toxicity).

In Tables 9 to 11, are shown results of lipids extraction from shark liver tissues. There is no marked difference in results between techniques within a species.

Tables 12 shows some characteristics features of fraction I (acetone) and fraction II (alcohol or ethyl acetate) for krill oil (e. pacifica). First, the saponification index of fraction I (130,6) indicates that this fraction contains fatty acids with longer chains, compared to fraction II (185,7). The Wijs iodine index of fraction I shows that this fraction contains high levels of polyunsaturated fatty acids. As compared to olive oil which has an index of 81.1. It explains why fraction I is liquid at room temperature. It is well known that unsaturated fatty acids have a fusion point inferior to the one of their saturated homologues. The same observations are made for fraction II which has a iodine index of 127,2. The fatty acid composition shown in Table 14 corroborates these iodine indexes: fraction I has a high percentage (30,24%) of polyunsaturated fatty acids (pentaenes+hexaenes) and so fraction II (22,98%). Finally, Table 12 shows also that fraction I is comprised of 10,0% of volatile matter and humidity after evaporation of the solvent. For the same test, the fraction II gives a value of 6,8%. To get rid of traces of solvents, it is important to briefly heat (to about 125°C, for about 15 min) the oil under nitrogen.

Results on krill oils obtained in accordance with the method of the present invention (fraction I extracted with acetone and fraction II extracted with ethyl acetate) are provided in Tables 12, 13, 14, 15, 16 and 17. It is noteworthy to mention that in Table 17, the carotenoids content was significantly high as measured in terms of two carotenoids namely astaxanthin and canthaxanthin. Indeed, duplicates analyzes revealed values of 92 to 124 µg/g of lipid fraction for astaxanthin and 262 to 734 µg/g for canthaxanthin. Thus, for the purpose of the present invention it may be said that the krill extract comprises astaxanthin at least 75 and preferably at least 90 ug/g of lipid fraction. In the case of canthaxanthin, at least 250 and preferably at least 270 µg/g of lipid fraction. Low values for peroxide and anisidine are advantageous and are due to the presence of high levels of natural antioxidants (astaxanthin and

canthaxanthin). These compounds are indicative of favourable pharmaceutical or cosmetological properties of the krill extract whereby high levels of carotenoids indicate excellent transdermal migration characteristics. Thus, krill extract is a good candidative for transdermal delivery of medicines.

Table 18 shows the best mode of the method in accordance with the present invention for lipid extraction of aquatic animal tissues.

Table 19 shows that the enzyme activity of the solid fraction is maintained following the method of the present invention. Indeed, the demonstration was completed for solid krill residue obtained after successive acetone and ethyl acetate extraction. Proteolytic activities were measure by the liberation of amino groups by spectrophotometric assay using *o*-pthaldialdehyde as reagent. Protein concentrations were measured by the Bradford method. Soluble proteins were extracted with water and added to a 10% lactoserum protein concentrate obtained by ultrafiltration. At the end of incubation at 37°C in 50mM potassium phosphate buffer, trichloroacetic acid was added and the amount of NH_3 group was measured in the supernatant according to the method of Church et al. [1983, J Dairy Sci 66: 1219-1227].

Figures 1 to 6 show chromatograms of fatty acid composition of *E. pacifica* lipids. On each of them, high proportions of 20:5 and 22:6 fatty acids (characteristic of marine and aquatic oils) are noticeable and represented by two distinct peaks.

Variations in lipid patterns of neutral lipids (from Figure 7 to Figure 11) from one species to another are attributable to the differences in food sources. Within a species (*E. pacifica*, for example) there is no marked variation between lipid patterns obtained from different techniques of lipid extraction. Concerning phospholipids (Figure 12 to Figure 16), the opposite is observed: variations are explained by the different extraction processes of lipids since the same species do not lead to the same lipid pattern. Lipids from shark species (extracted by the mentioned methods)

and commercial cod-liver oil (sample available from Uniprix drugstores, Province of Québec, Canada) are mainly composed of neutral lipids as opposed to phospholipids.

The influence of the volume of solvent and incubation time on the efficiency of the acetone to extract lipids from *E. pacifica* is illustrated in Figures 17 and 18, respectively. A ratio of 1:6 (w/v) produced optimal yield with near complete extraction after 2h. The second extraction step has been experimented with ethanol. The volume of this solvent does not appear to be critical since the same yield was obtained with different volumes of ethanol (Figure 19), but incubations time in ethanol should be at least 30 minutes as indicated by the results on Figure 20.

One of the inventors, Dr. Adrien Beaudoin, has ingested the different lipid fractions of krill. No side effect profile was observed.

Although the invention has been described above with respect with one specific form, it will be evident to a person skilled in the art that it may be modified and refined in various ways. It is therefore wished to have it understood that the present invention should not be limited in scope, except by the terms of the following claims.

Demonstration that krill residue, obtained after acetone and ethyl acetate extraction, contains enzyme proteolytic activities. Proteolytic activities were measured by the liberation of amino groups by spectrophotometric assay using *o*-phthaldialdehyde as reagent. Protein concentrations were measured by the Bradford method.

The enzyme source was the residue obtained after acetone and ethyl acetate extractions of lipids. Soluble proteins were extracted with water and added to a 10% lactoserum protein concentrate obtained by ultrafiltration.

At the end of incubation at 37°C in 50 mM potassium phosphate buffer, trichloroacetic acid was added and the amount of NH_3 groups were measured in the supernatant according to Church and al. 1983.

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TABLE 1. EXTRACTION OF DRY KRILL LIPIDS (*E. pacifica*)

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
1-	acetone [a]	8,00		
	ethanol [b]	7,60	**15,60**	
2-	"	19,70		
		6,90	**26,60**	
3-	"	8,15		
		11,20	**19,35**	
4-	"	6,80		
		13,60	**20,40**	
				20,49±3,95
5-	chlor : MeOH [c]		**15,50**	
6-	"		**14,90**	
				15,20±0,30

Determinations in triplicates (variation < 5 %).
[a] :Extraction made with a sample-solvent ratio of 1:9 (w/v), no incubation.
[b] :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 night at 4°C, following a first extraction with acetone.
[c] :Folch et al. 1957.

TABLE 2. EXTRACTION OF FROZEN KRILL LIPIDS (*E. pacifica*)

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
1-	acetone [a]	1,17		
	ethanol [b]	1,23	**2,40**	
2-	"	3,05		
		1,09	**4,14**	
3-	"	1,53		
		1,26	**2,79**	
				3,11±0,91
4-	acetone [a]	2,45		
	isopropanol [b]	0,70	**3,15**	
5-	"	1,80		
		0,80	**2,60**	
6-	"	1,60		
		0,80	**2,40**	
				2,72±0,39

TABLE 2 (continued). EXTRACTION OF FROZEN KRILL LIPIDS (*E. pacifica*)

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
7-	acetone [a]	2,15		
	t-butanol [c]	0,47	**2,62**	
8-	"	2,11		
		0,40	**2,51**	
9-	"	2,37		
		0,45	**2,82**	
				2,65±0,16
10-	acetone [a]	2,28		
	ethyl acetate [b]	0,21	**2,49**	
11-	"	1,09		
		0,16	**1,25**	
12-	"	2,54		
		0,09	**2,63**	
				2,12±0,76
13-	combined acetone-ethanol [d]		**3,28**	
14-	"		**3,02**	
15-	"		**3,25**	
				3,18±0,14
16-	ethyl acetate [e]		**1,32**	
17-	"		**1,49**	
18-	"		**1,31**	
				1,37±0,10
19-	hexane [e]		**0,31**	
20-	"		**0,18**	
21-	"		**0,20**	
				0,23±0,07
22-	chlor:MeOH [f]		**2,37**	

TABLE 2 (continued). EXTRACTION OF FROZEN KRILL LIPIDS (*E. pacifica*)

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
23-	"		**2,07**	
24-	"		**2,62**	
				2,35±0,28

Determinations in triplicates (variation < 5 %).
[a] :Extraction made with a sample-solvent ratio of 1:6 (w/v), incubated 2 h at 4°C.
[b] :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.
[c] :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 25°C, following a first extraction with acetone.
[d] :Extraction made with a sample-acetone-ethanol ratio of 1:5:5 (w/v/v), incubated 2 h at 4°C.
[e] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 2 h at 4°C.
[f] : Folch et al. 1957.

TABLE 3. EXTRACTION OF FROZEN KRILL LIPIDS(M. norvegica)

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
1-	acetone [a]	1,82		
	ethanol [b]	1,82	**3,64**	
2-	"	1,15		
		2,35	**3,50**	
3-	"	1,68		
		2,19	**3,87**	
				3,67±0,15

Determinations in triplicates (variation < 5 %).
[a] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 1 night at 4°C.
[b] :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 h at 4°C, following a first extraction with acetone.

TABLE 4. INFLUENCE OF GRINDING ON EXTRACTION OF FROZEN KRILL LIPIDS (M. norvegica)

Exp. No.	Technique	Krill ground before 1st extraction	Yield (%)	Total (%)
1-	acetone [a]	yes	3,10	
	ethanol [b]		1,07	4,17
2-	"	no	2,14	
			1,39	3,53
3-	"	yes	3,32	
			1,14	4,46
4-	chlor : MeOH [c]	yes		3,30
5-	"	yes		3,26

Determinations in triplicates (variation < 5 %).

[a] :Extraction made with a sample-solvent ratio of 1:6, incubated 2 h at 4°C.

[b] :Extraction made with a sample-solvent ratio of 1:2, incubated 30 min at 4°C, following a first extraction with acetone.

[c] :Folch et al. 1957.

TABLE 5. EXTRACTION OF FROZEN *Calanus* LIPIDS (*Calanus* sp.)

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) ± s.d.
1-	acetone [a]	6,18		
	ethanol [b]	2,04	**8,22**	
2-	"	8,64		
		2,26	**10,90**	
				9,56±1,34

Determinations in triplicates (variation < 5 %).

[a] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 1 night at 4°C.

[b] :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 h at 4°C, following a first extraction with acetone.

TABLE 6. EXTRACTION OF FRESH FISH LIPIDS (Mackerel)

Exp. No.	Technique	Yield (%)	Total (%)
1- viscera	acetone [a]	6,11	
fish 1	ethanol [b]	0,59	**6,70**
2- tissues	"	3,78	
fish 1		0,91	**4,69**
3- viscera	"	10,46	
fish 2		0,57	**11,03**
4- issues	"	6,65	
fish 2		1,41	**8,06**
5- viscera	"	8,39	
fish 3		0,66	**9,05**
6- tissues	"	5,27	
fish 3		0,97	**6,24**
7- viscera	"	8,47	
fish 4		0,69	**9,16**
8- tissues	"	8,40	
fish 4		1,02	**9,42**
9- viscera fish 1	chlor:MeOH [c]		**0,52**
10- tissues fish 1	"		**1,45**

[a] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubation time:

- fish 1 viscera: 4h, fish 1 tissues: 23h
- fish 2 viscera: 23h45, fish 2 tissues: 45h30
- fish 3 viscera: 8 days 2h20, fish 3 tissues: 8 days 22h30
- fish 4 viscera: 17 days 23h, fish 4 tissues: 18 days 2h25.

[b] :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1h at 4°C, following a first extraction with acetone.

[c] :Folch et al. 1957, following extractions with acetone, then ethanol..

TABLE 7. EXTRACTION OF FRESH FISH LIPIDS (Trout)

Exp. No.	Technique	Yield (%)	Total (%)
1- viscera	acetone [a]	34,70	
	ethanol [b]	2,18	**36,88**
2- tissues	"	5,53	
		1,17	**6,70**
3- viscera	chlor:MeOH [c]		**39,81**
4- tissues	"		**14,93**

Determinations in triplicates (variation < 5 %).

[a] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 1 night at 4°C.

[b] :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 h at 4°C, following a first extraction with acetone.

[c] :Folch et al. 1957.

TABLE 8. EXTRACTION OF FRESH FISH LIPIDS (Herring)

Exp. No.	Technique	Yield (%)	Total (%)
1-tissues and viscera	acetone [a]	2,09	
	ethanol [b]	0,68	**2,77**
2-tissues and viscera	chlor:MeOH [c]		**5,95**

Determination in triplicates (variation < 5 %).

[a] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 1 night at 4°.

[b] :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 h at 4°C, following a first extraction with acetone.

[c] :Folch et al. 1957.

TABLE 9. EXTRACTION OF FRESH SHARK LIVER LIPIDS (M. schmitti)

Exp. No.	Technique	Yield (%)	Total (%)
1-	acetone [a]	36,39	
	ethyl acetate [b]	4,48	**40,87**
2-	ethyl acetate [c]		**36,68**
3-	chlor : MeOH [d]		**41,86**

Determinations in triplicates (variations <5 %).

[a] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 2h at 4°C.

[b] :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

[c] :Extraction made with a sample-solvent ratio of 1 :9 (w/v), incubated 2h at 4°C.

[d] :Folch et al. 1957.

a first extraction with acetone.
[c] :Extraction made with a sample-solvent ratio of 1 :9 (w/v), incubated 2h at 4°C.
[d] :Folch et al. 1957.

TABLE 10. EXTRACTION OF FRESH SHARK LIVER LIPIDS (G. galeus).

Exp. No.	Technique	Yield (%)	Total (%)
1-	acetone [a]	21,39	
	ethyl acetate [b]	5,27	**26,66**
2-	ethyl acetate [c]		**25,89**
3-	chlor : MeOH [d]		**29,99**

Determinations in triplicates (variations <5 %).
[a] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 2h at 4°C.
[b] :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.
[c] :Extraction made with a sample-solvent ratio of 1 :9 (w/v), incubated 2h at 4°C.
[d] :Folch et al. 1957.

TABLE 11. EXTRACTION OF FRESH SHARK LIVER LIPIDS (Angel Shark)

Exp. No.	Technique	Yield (%)	Total (%)
1-	acetone [a]	19,23	
	ethyl acetate [b]	8,98	**28,21**
2-	ethyl acetate [c]		**39,22**
3-	chlor : MeOH [d]		**39,23**

Determinations in triplicates (variations <5 %).
[a] :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 2h at 4°C.
[b] :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.
[c] :Extraction made with a sample-solvent ratio of 1 :9 (w/v), incubated 2h at 4°C.
[d] :Folch et al. 1957.

TABLE 12. CHARACTERISTICS OF KRILL OIL (*E. pacifica*)

		independent laboratory [a]	handbook[b]
Saponification index			
Fraction I [c]	130,6	---	---
Fraction II [d]	185,7	---	---
Olive oil	192,0 [e]	---	189,7
Wijs iodine index			
Fraction I [c]	185,2	172,5	---
Fraction II [d]	127,2	139,2	---
Olive oil	85,3 [e]	---	81,1
Cholesterol content (%)			
Fraction I [c]	2,1	1,9	---
Fraction II [d]	3,7	3,0	---
Olive oil	0,2 [e]	---	---
Volatile matter and moisture levels (%)			
Fraction I [c]	10,0	---	---
Fraction II [d]	6,8	---	---
Peroxide value (meq peroxide/kg oil)			
Fraction I [c]	---	0,0	---
Fraction II [d]	---	0,0	---
p-Anisidine value (g^{-1} absorption)			
Fraction I [c]	---	0,1	---
Fraction II [d]	---	5,5	---

[a]: Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.
[b]: Harwood and Geyer 1964.
[c]: Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.
[d]: Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.
[e]: Extra virgin olive oil cold compressed from Bertolli ™.

TABLE 13. LIPID CLASS COMPOSITION OF KRILL OIL (AREA %) (*E. pacifica*)

Triglycerides	
Fraction I [a]	19,0±0,7
Fraction II [b]	66,5± 2,3
Hydrocarbons	
Fraction I [a]	trace
Fraction II [b]	1,3± 0,1
Free fatty acids	
Fraction I [a]	23,7± 1,1
Fraction II [b]	20,3± 0,3
Monoglycerides	
Fraction I [a]	1,4± 0,3
Fraction II [b]	0,5± 0,1
Phospholipids or other polar material	
Fraction I [a]	54,1± 6,1
Fraction II [b]	8,5 ±1,6

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.

[a] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.

[b] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

TABLE 14. FATTY ACID COMPOSITION OF KRILL OIL (WT/WT%) (*E. pacifica*)

Fatty acids	Fraction I [a)]	Fraction II [b)]
12 :0	0,0	0,1
13 :0	0,2	0,1
ISO 14 :0	0,4	0,6
14:0	4,2	7,6
ISO 15:0	0,5	0,7
ANT 15:0	0,2	0,2
15:0	0,6	1,0
ISO 16:0	0,2	0,3
ANT 16:0	0,2	0,2
16:0	14,1	21,6
7MH	0,6	0,9
ANT 17:0	0,1	0,3
17:0	2,8	3,7
18:0	1,0	1,6
20:0	0,1	0,3
Saturates	25,2	39,2

TABLE 14 (continued). FATTY ACID COMPOSITION OF KRILL OIL (WT/WT%) (*E. pacifica*)

Fatty acids	Fraction I [a)]	Fraction II [b)]
14:1	0,4	0,5
15:1	0,1	0,2
16:1 n-7	6,6	7,8
16:1 n-5	0,6	0,2
17:1	0,6	0,7
18:1 n-9	8,0	9,8
18:1 n-7	4,2	5,6
18:1 n-5	0,1	0,1
20:1 n-9	0,3	0,4
20:1 n-7	0,3	0,4
20:1 n-5	0,3	0,4
22:1 n-11 +13	0,1	0,2
Monoenes	21,6	26,3
16:2 n-6	0,6	1,2
16:2 n-4	1,3	1,3
18:2 n-7	0,1	0,2
18:2 n-6	2,0	1,8
18:2 n-4	0,1	0,1
20:2 NMID	0,2	0,2
20:2 n-6	0,1	0,1
Dienes	4,4	4,9
16:3 n-4	1,4	1,2
18:3 n-6	0,4	0,3
18:3 n-4	0,2	0,2
18:3 n-3	3,2	3,0
18:3 n-1	0,1	0,1
20:3 n-3	0,1	0,1
Trienes	5,4	4,9
16:4 n-3	0,9	0,7
16:4 n-1	1,0	0,8
18:4 n-3	9,2	7,4
18:4 n-1	0,1	0,0
20:4 n-6	0,7	0,5
20:4 n-3	0,7	0,3
Tetraenes	12,6	9,7
20:5 n-3	17,4	8,6
21:5 n-3	0,7	0,5
22:5 n-6	0,2	0,1
22:5 n-3	0,5	0,3
Pentaenes	18,8	9,5

TABLE 14 (continued). FATTY ACID COMPOSITION OF KRILL OIL (WT/WT%) (*E. pacifica*)

Fatty acids	Fraction I [a)]	Fraction II [b)]
22:6 n-3	13,2	6,6
Hexaenes		
Iodine value calculated	214,8	145,1

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.

[a)] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.

[b)] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

TABLE 15. KRILL LIPID FREE FATTY ACID FAME (WT/WT%) (*E. pacifica*)

Fatty acids	Fraction I [a)]	Fraction II [b)]
12:0	0,5	0,1
13:0	0,2	0,0
ISO14:0	0,2	0,2
14:0	1,3	2,6
ISO 15:0	0,3	0,3
ANT 15:0	0,1	0,1
15:0	0,2	0,5
ISO 16:0	0,1	0,2
ANT 16:0	0,2	0,1
16:0	3,3	10,6
7MH	0,6	0,8
ANT 17:0	0,2	0,2
Phytanic	0,2	0,0
17:0	0,5	0,8
18:0	0,2	0,6
20:0	0,3	0,2
22:0	0,0	0,1
Saturates	8,4	17,4
14:1	0,2	0,2
15:1	0,2	0,1
16:1 n-9	0,5	0,0
16:1 n-7	5,2	6,8
16:1 n-5+I17:0	0,1	0,1
17:1	0,6	0,7
18:1 n-9	7,0	11,4
18:1 n-7	4,9	9,3
18:1 n-5	0,1	0,3
20:1 n-11	0,2	0,3
20:1 n-9	0,1	0,3

22:1 n-11+13	0,1	0,2
24:1 n-9	0,0	0,1
Monoenes	19,2	29,8

TABLE 15 (continued). KRILL LIPID FREE FATTY ACID FAME (WT/WT%) (*E. pacifica*)

Fatty acids	Fraction I [a]	Fraction II [b]
16:2 n-6	0,4	0,9
16:2 n-4	1,2	1,0
18:2 n-7	0,1	0,2
18:2 n-6	2,4	2,6
18:2 n-4	0,1	0,1
20:2 n-6	0,1	0,1
Dienes	4,3	4,9
16:3 n-4+I17:1	1,4	0,9
16:3 n-3+I18:0	0,2	0,5
18:3 n-6	0,4	0,3
18:3 n-4	0,1	0,1
18:3 n-3	3,3	3,4
18:3 n-1	0,1	0,1
20:3 n-6	0,1	0,1
20:3 n-3	0,1	0,2
Trienes	5,7	5,6
16:4 n-3	0,6	0,3
16:4 n-1	1,0	0,6
18:4 n-3	9,8	6,2
18:4 n-1	0,1	0,1
20:4n-6	1,7	1,4
20:4 n-3	0,6	0,5
22:4 n-3	0,3	0,3
Tetraenes	14,1	9,4
18:5 n-3	0,2	0,1
20:5 n-3	26,4	17,4
21:5 n-3	0,9	0,6
22:5 n-6	0,0	0,1
22:5 n-3	0,7	0,5
Pentaenes	28,2	18,7
22:6 n-3	20,5	14,4
Hexaenes	20,5	14,4
Iodine value calculated	291,6	220,3

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.

[a] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.

[b] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

TABLE 16. TOCOPHEROL, ALL-*trans* RETINOL AND CHOLECALCIFEROL CONTENT IN KRILL OIL (*E. pacifica*)

alpha-tocopherol by HPLC (IU)	
Fraction I [a)]	0,91
Fraction II [b)]	0,83
gamma-tocopherol by HPLC µg/g	
Fraction I [a)]	Tr
Fraction II [b)]	Tr
delta-tocopherol by HPLC µg/g	
Fraction I [a)]	N.D.
Fraction II [b)]	N.D.
all-*trans* retinol by HPLC (IU)	
Fraction I [a)]	395,57
Fraction II [b)]	440,47
cholecalciferol by HPLC (IU)	
Fraction I [a)]	N.D.
Fraction II [b)]	N.D.

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.
Data expressed per gram of krill oil.
[a)] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.
[b)] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.
TR = trace
N.D. = not detected

Conversion : Vitamin	alpha-tocopherol	mg/g oil x 1,36 = International Unit
	All-*trans* retinol	µg/g ÷ 0,3 = International Unit

TABLE 17. ASTAXANTHIN AND CANTHAXANTHIN CONTENT OF KRILL OIL (*E. pacifica*)

Asthaxantin (µg/g oil)	
Fraction I [a)]	93,1
Fraction II [b)]	121,7
Canthaxanthin (µg/g oil)	
Fraction I [a)]	270,4
Fraction II [b)]	733,0

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.
[a)] : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.
[b)] : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

TABLE 18. OPTIMAL CONDITIONS FOR LIPID EXTRACTION OF AQUATIC ANIMAL TISSUES (suggested procedure)

STEP	CONDITIONS
Grinding (if particles > 5mm)	4°C
Lipid extraction	sample-acetone ratio of 1:6 (w/v) 2h (including swirling 20 min) 4°C
Filtration	organic solvent resistant filter under reduced pressure
Washing	sample-acetone ratio of 1:2 (w/v) pure and cold acetone
Filtration	organic solvent resistant filter under reduced pressure
Evaporation	under reduced pressure
Oil-water separation	4°C
Lipid extraction	sample: ethyl acetate ratio of 1:2 (w/v)[a)] pure ethyl acetate 30 min 4°C [b)]
Filtration	organic solvent resistant filter under reduced pressure
Evaporation	under reduced pressure

[a)]: Ethanol can be replaced by isopropanol, *t*-butanol or ethyl acetate.
[b)]: 25 °C when using *t*-butanol.

TABLE 19: PROTEOLYTIC ACTIVITY OF KRILL RESIDU USING LACTOSERUM AS THE SUBSTRATE, AT 37 °C, PH 7.0 FOR A RATIO ENZYME:SUBSTRATE OF 1:43

Time (min)	Amino acids released (µmoles)	Enzymatic rate (µmoles/min)	Specific enzymatic activity (µmoles/min/mg*)
15	28.76	1.917	0.164
30	43.74	0.999	0.125
170	98.51	0.322	0.050
255	177.26	0.308	0.060

* total quantity of enzymes in hydrolysis media

CLAIMS

1. A method for extracting lipid fractions from marine and aquatic animal material, said method comprising the steps of:

(a) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents;

(c) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(d) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(e) separating the liquid and solid contents;

(f) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(g) recovering the solid contents.

4. A method as in claim 1, wherein steps (b) and (d) are conducted under inert gas atmosphere.

5. A method as in claim 1, wherein steps (b) and (e) are effected by techniques selected from filtration, centrifugation and sedimentation.

6. A method as in claim 1 , wherein steps (c) and (f) are effected by techniques selected from vacuum evaporation, flash evaporation and spray drying.

7. A method as in claim 1, wherein after step (b) and before step (c), the method

additionally comprises the intervening step of washing the solid contents with the solvent and adding the resulting washing solution to the liquid contents of step (b).

8. A method as in claim 1, wherein after step (e) and before step (f), the method additionally comprises the intervening step of washing the solid contents with the organic solvent selected in step (d).

9. A method as in claim 1, wherein prior to step (a) the marine and aquatic animal material is finely divided.

10. A method as in claim 1, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

11. A method as in claim 1, wherein said marine and aquatic animal is zooplancton.

12. A method as in claim 11, wherein said zooplancton is selected from krill and *Calanus*.

14. A method as in claim 1, wherein said marine and aquatic animal is fish filleting by-products.

15. A method for extracting an astaxanthin-and-canthaxantin-containing lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, said method comprising the steps of:

(a) placing said animal material in a ketone solvent to achieve an extraction of the soluble lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents;

(c) recovering a lipid-rich fraction from the liquid contents by evaporation of the solvent present in the liquid contents;

whereby an astaxanthin-and-canthaxantin-containing lipid fraction is obtained.

16. A method for extracting a lipid fraction from a marine and aquatic animal material selected from zooplancton and fish filleting by-products, said method comprising the steps of:

(a) placing said animal material in a solvent mixture comprising acetone and ethanol to achieve an extraction of the soluble lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents;

(c) recovering a lipid-rich fraction from the liquid contents by evaporation of the solvents present in the liquid contents;

whereby a lipid fraction is obtained.

17. A method as in claim 15, wherein the animal material is selected from krill and *Calanus*.

19. A method as in claim 15, wherein during step (a), the animal material is homogenized.

20. A method as in claim 15, wherein steps (b) and (d) are conducted under inert gas atmosphere.

21. A method as in claim 15, wherein step (b) is effected by a technique selected from filtration, centrifugation and sedimentation.

22. A method as in claim 15, wherein step (c) is effected by a technique selected from vacuum evaporation, flash evaporation and spray drying.

23. A method as in claim 15, wherein after step (b) and before step (c), the method

additionally comprises a step of washing said solid contents with solvent and adding the resulting washing solution to the liquid contents of step (b).

24. A method as in claim15, wherein prior to step (a) the marine and aquatic animal material is finely divided.

25. A method as in claim 15, wherein steps (a) and (b) are conducted at solvent temperatures of not more than about 5°C.

26. A krill lipid extract characterized in that the carotenoid content in asthaxanthin is at least about 75 mg/g of krill extract, and the carotenoid content in canthaxanthin is at least about 250 mg/g of krill extract.

27. A method of lipid extraction as in claim 1, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

30. A method of lipid extraction as in claim 15, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

31. A method for extracting lipid fractions from marine and aquatic animal material, said method comprising the steps of:

(a) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(b) separating the liquid and solid contents;

(c) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(d) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction

of the remaining soluble lipid fraction from said marine and aquatic animal material;

(e) separating the liquid and solid contents:

(f) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

whereby lipid fractions are obtained.

32. A method of lipid extraction as in claim 31, wherein the solid contents of step (b) is recovered and consists of a dehydrated residue containing active enzymes.

33. A lipid fraction extracted from marine and aquatic animal material, by a method comprising the steps of:

(g) placing marine and aquatic animal material in a ketone solvent to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;

(h) separating the liquid and solid contents;

(i) recovering a first lipid-rich fraction from the liquid contents of step (b) by evaporation of the solvent present in the liquid contents;

(j) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;

(k) separating the liquid and solid contents;

(l) recovering a second lipid-rich fraction by evaporation of the solvent from the liquid contents of step (e);

(m) recovering the solid contents.

36. A method as in claim 1, wherein the ketone solvent is acetone.

37. A method as in claim 1, wherein the alcohol is selected from the group of ethanol, isopropanol and *t*-butanol.

38. A method as in claim 1, wherein the ester of acetic acid is ethyl acetate.

39. A method as in claim 9, wherein the marine and aquatic animal material is finely divided to an average particle size of not more than 5mm.

40. A method as in claim 15, wherein said marine and aquatic animal material is viscera.

41. A method as in claim 15, wherein the ketone solvent is acetone.

42. A method as in claim 16, wherein said marine and aquatic animal material is viscera.

43. A method as in claim 16, wherein the animal material is selected from krill and *Calanus*.

44. A method as in claim 24, wherein the animal material is finely divided to an average particle size of not more than 5mm.

45. A krill lipid extract as in claim 26, wherein the carotenoid content in asthaxanthin is at least about 90 mg/g of krill extract.

46. A krill lipid extract as in claim 26, wherein the carotenoid content in canthaxanthin is at least about 270 mg/g of krill extract.

47. A method as in claim 1, wherein the solid contents of step (e) is recovered and

consists of a dehydrated residue containing active enzymes.

48. A method as in claim 31, wherein the ketone solvent is acetone.

49. A method as in claim 31, wherein the alcohol is selected from the group of , ethanol, isopropanol and *t*-butanol.

50. A method as in claim 31, wherein the ester of acetic acid is ethyl acetate.

51. A method of lipid extraction as in claim 31, wherein the solid contents of step (e) is recovered and consists of a dehydrated residue containing active enzymes.

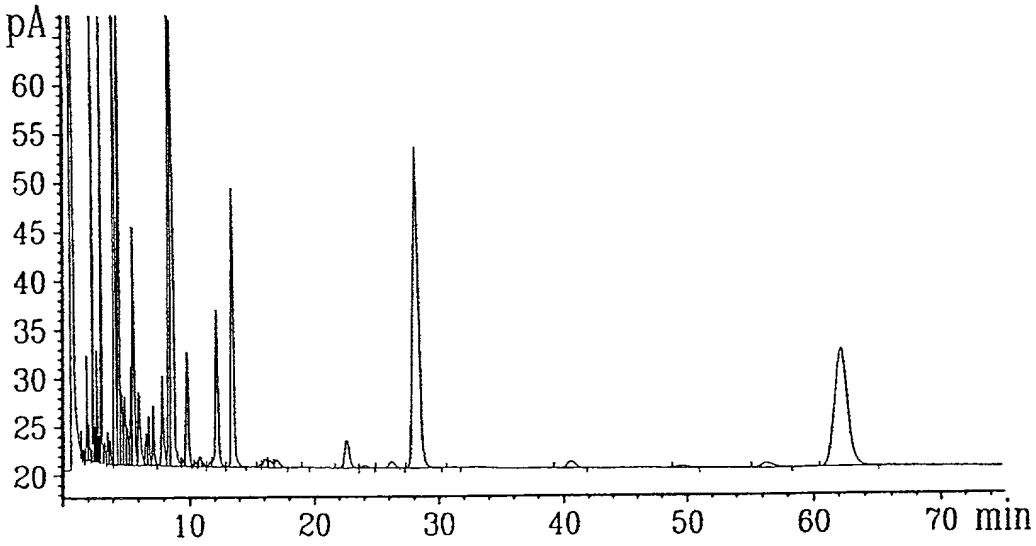


Fig. 1

1.263	4.521 - 16:1	11.637
1.455 - 12:0	4.684	12.145 - 18:3
1.625	4.891 - 16:1tr	13.458
1.812	5.121	15.626 - 20:0
1.876	5.426	16.045
2.056	5.570	16.482 - 20:1
2.173	6.037	17.017 - 20:1(cis11)
2.331 - 14:0	6.662	19.344 - 20:2
2.505	6.871	22.606 - 20:4(6,10,14,18)
2.591 - 14:1	7.235	24.103
2.682	7.925 - 18:0	26.247
2.802	8.439 - 18:1	28.287
2.855	8.640 - 18:1tr	31.295
3.078 - std 15:0	9.544	40.655
3.309	9.801 - 18:2	49.721
3.586	10.491	56.373
3.810	10.825	62.225
4.176 - 16:0	11.042	

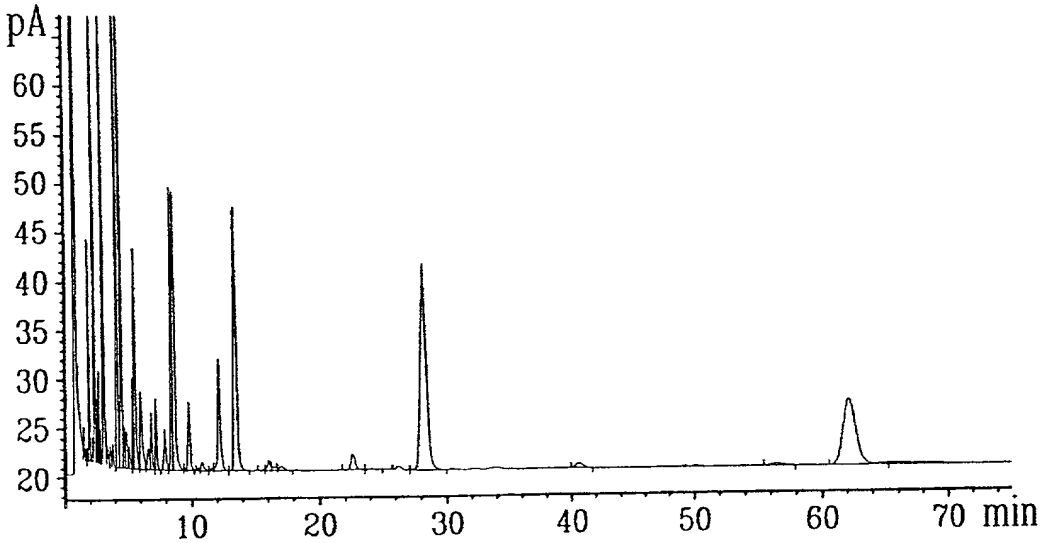


FIG. 2

1.217	3.806	9.786 - 18:2
1.264	4.157 - 16:0	10.484
1.454 - 12:0	4.515 - 16:1	10.813
1.624	4.680	11.590
1.812	4.891 - 16:1tr	12.136 - 18:3
1.876	5.028	13.447
2.055	5.109	15.623 - 20:0
2.171	5.421	16.025
2.330 - 14:0	5.562	16.466 - 20:1
2.505	6.031	17.021 - 20:1 (cis11)
2.591 - 14:1	6.642	22.585 - 20:4 (6,10,14,18)
2.680	6.870	24.100
2.800	7.230	26.217
2.854	7.910 - 18:0	28.241
3.077 - std 15:0	8.419 - 18:1	40.622
3.306	8.622 - 18:1tr	56.417
3.585	9.529	62.086

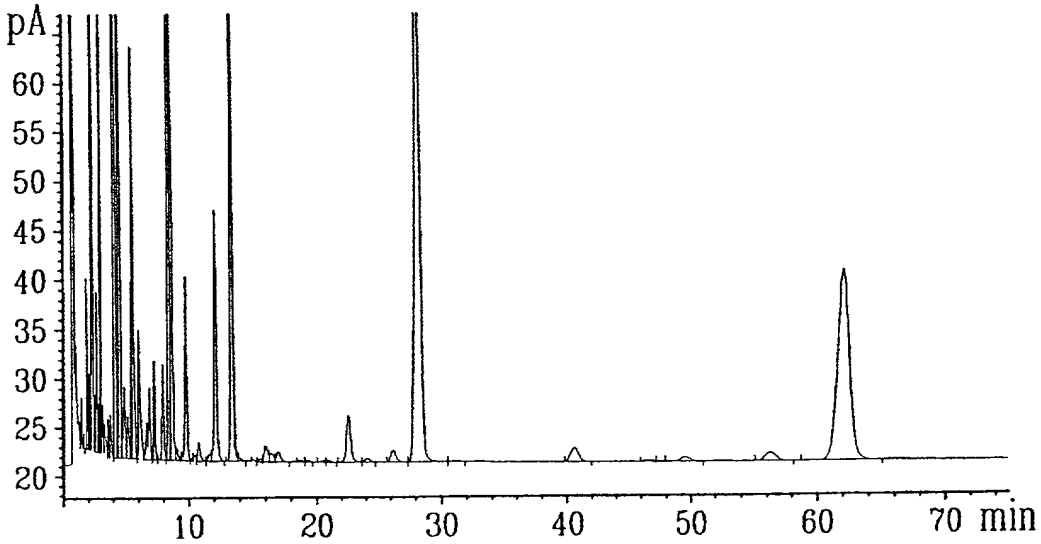


Fig. 3

1.216	4.520 - 16:1	13.457
1.262	4.683	13.943
1.454 - 12:0	4.884 - 16:1tr	15.053
1.624	5.030	15.572 - 20:0
1.811	5.111	16.016
1.875	5.420	16.486 - 20:1
2.016	5.561	16.999 - 20:1(cis11)
2.054	6.031	18.762
2.174	6.642	19.303 - 20:2
2.330 - 14:0	6.868	20.474
2.505	7.226	21.027 - 20:3
2.589 - 14:1	7.908 - 18:0	22.575 - 20:4 (6,10,14,18)
2.679	8.444 - 18:1	24.071
2.799	8.639 - 18:1tr	26.215
2.854	9.005	28.333
2.981	9.536	31.180
3.074 - std 15:0	9.788 - 18:2	40.560
3.304	10.267	46.595
3.580	10.481	49.513
3.804	10.807	56.292
4.169 - 16:0	11.626	62.250
4.296	12.140 - 18:3	

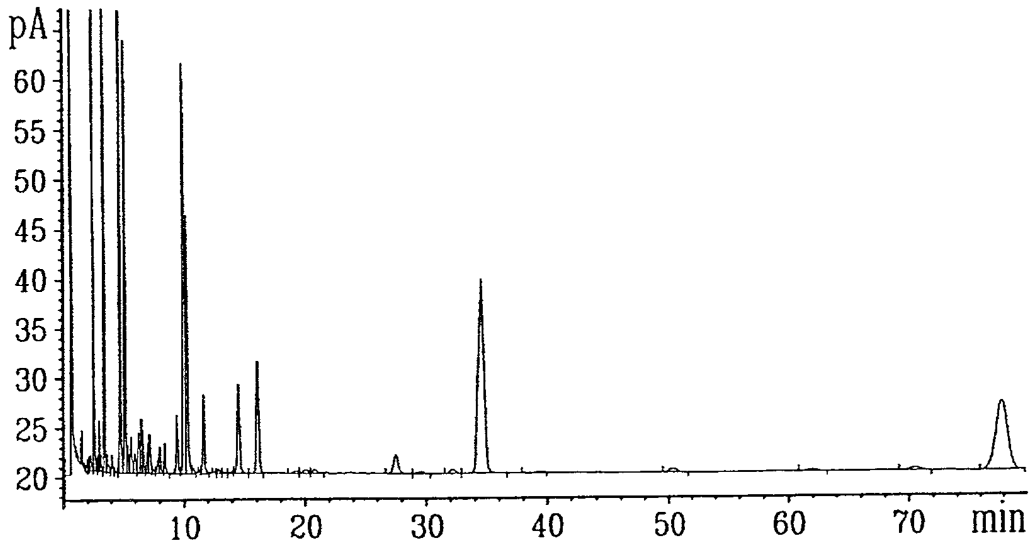


FIG. 4

1.552 - 12:0
1.749
1.968
2.095
2.262
2.485
2.582 - 14:0
2.784
2.886 - 14:1
3.004
3.145
3.478 - std 15:0
3.720
4.088
4.325
4.793 - 16:0
5.196 - 16:1
5.406
5.675 - 16:1tr
5.964
6.284
6.533
6.655
7.009
7.159
7.440
7.874
8.019
8.462
9.411 - 18:0
10.000 - 18:1
10.249 - 18:1tr
10.716
11.357
11.647 - 18:2
12.519
12.888
13.388
14.017
14.524 - 18:3
16.107
19.275 - 20:0
20.112 - 20:1 (cis11)
20.781 - 20:1
27.553
29.529
32.161
34.614
39.240
50.374
61.892
70.568
77.894

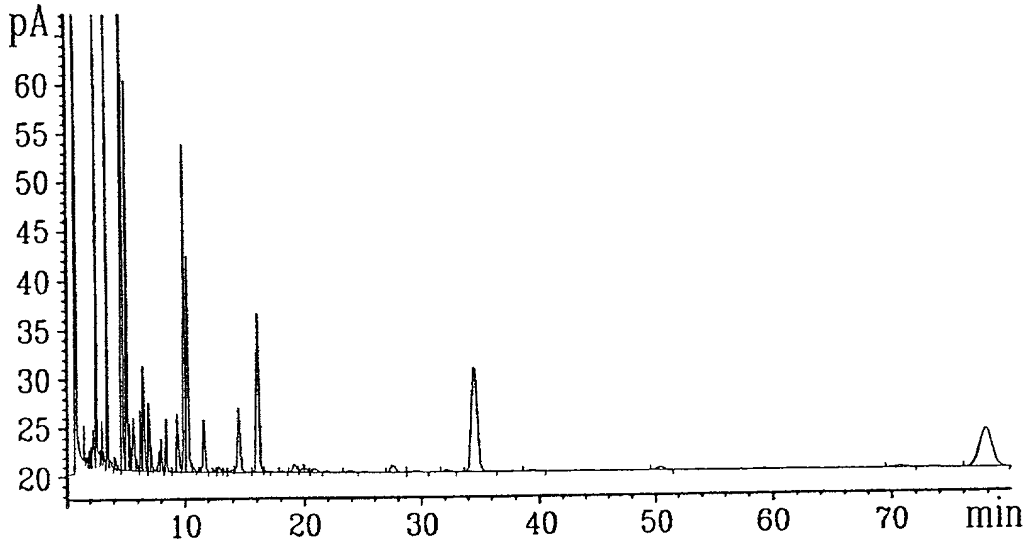


FIG. 5

1.556 – 12:0
1.753
1.972
2.104
2.304
2.590 – 14:0
2.892 – 14:1
3.012
3.153
3.485 – std 15:0
3.710
4.096
4.203
4.333
4.800 – 16:0
5.206 – 16:1
5.417
5.711 – 16:1tr
5.977
6.294
6.546
7.020
7.166
7.889
8.030
8.473
9.425 – 18:0
10.010 – 18:1
10.260 – 18:1tr
10.735
11.394
11.661 – 18:2
12.540
12.909
13.402
14.010
14.540 – 18:3
16.133
16.805
18.131
19.154 – 20:0
19.875
20.099 – 20:1(cis11)
20.820 – 20:1
23.903
27.583
29.570
32.195
34.597
39.334
50.452
70.660
77.895 – 24:0

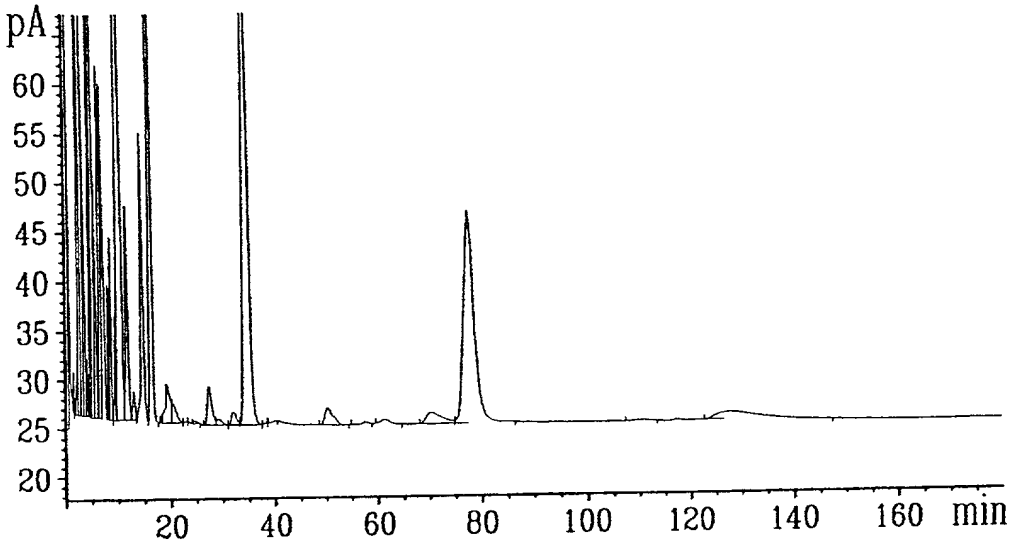


Fig 6

1.564 - 12:0	8.025	34.677
1.762	8.485	38.373 - 22:0
1.977	9.360 - 18:0	40.163
2.080	9.407	50.157
2.273	10.147 - 18:1tr	57.532
2.591 - 14:0	11.618 - 18:2	61.436
2.887 - 14:1	12.858	70.271
3.008	14.515 - 18:3	77.784 - 24:0
3.470 - std 15:0	16.162	110.694
4.108	18.077	127.696
4.341	19.355 - 20:1	
4.803 - 16:0	20.182 - 20:4 (cis11)	
5.210 - 16:1tr	20.311	
5.683	23.205 - 20:2	
6.292	24.678	
6.514	27.411 - 20:4 (6,10,14,18)	
7.030	29.307	
7.810	31.990	

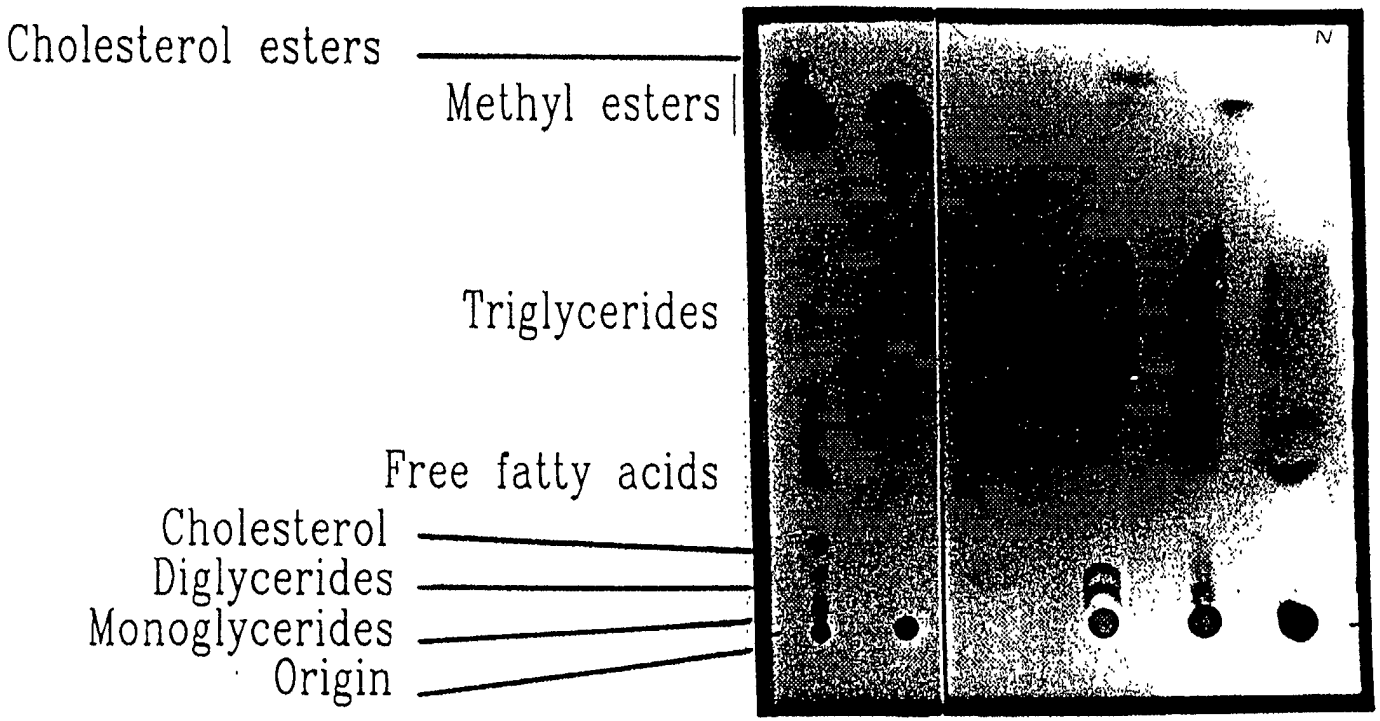


FIG. 7

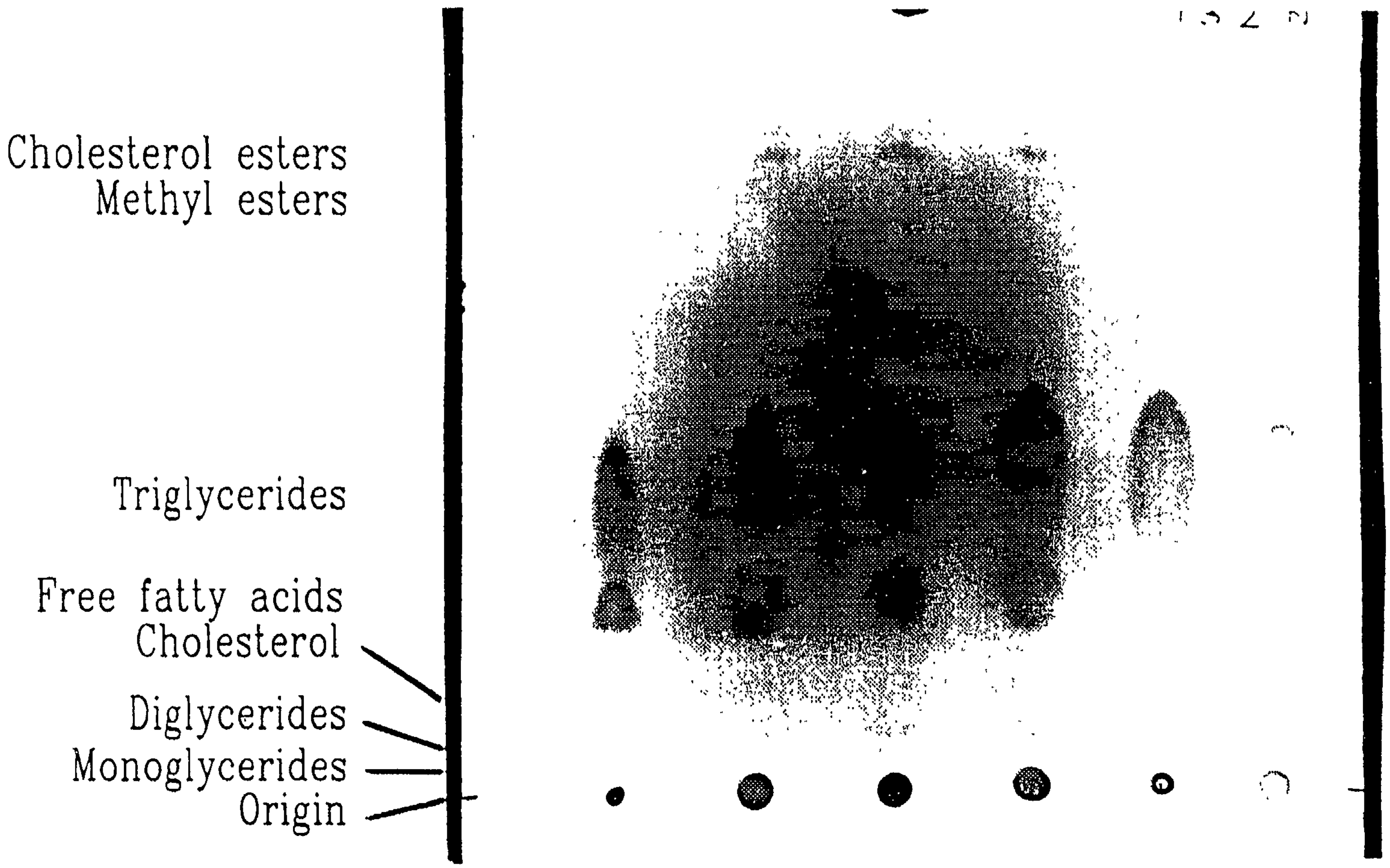
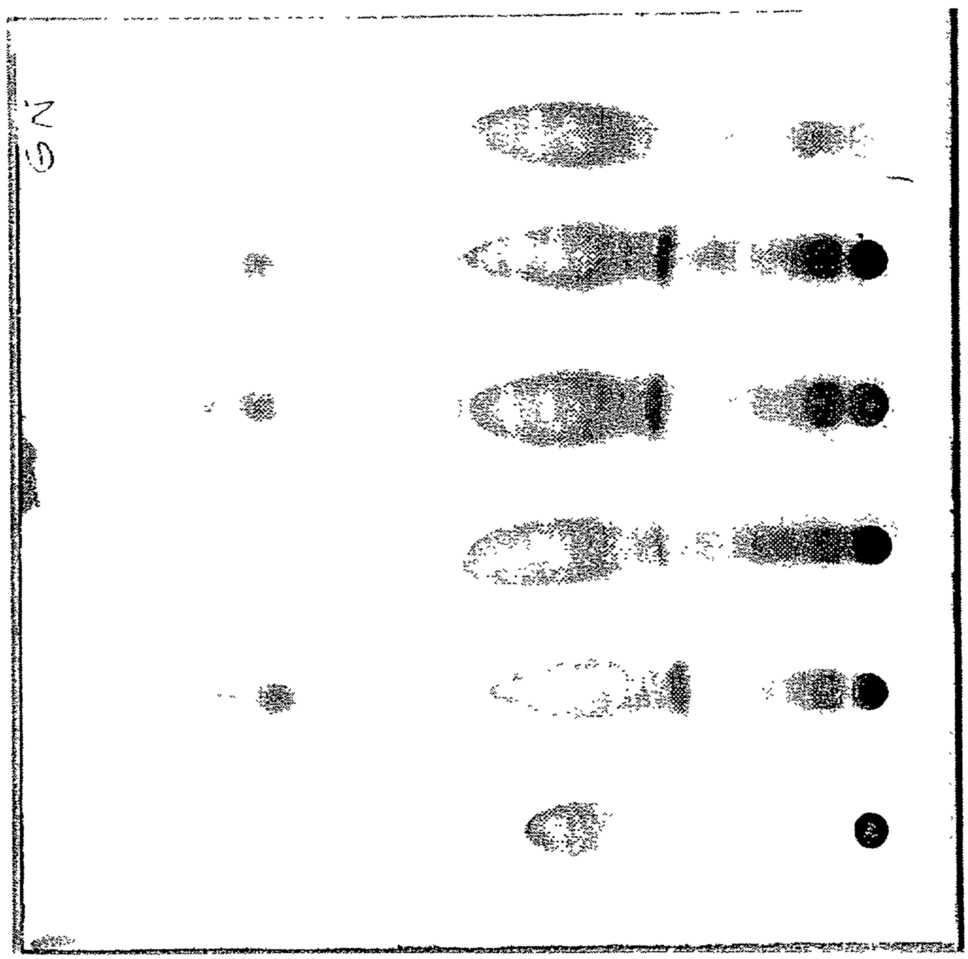

Cholesterol esters
Methyl esters
Triglycerides
Free fatty acids
Cholesterol
Diglycerides
Monoglycerides
Origin

FIG. 8

Fig. 6

09/830146



A B C D E F G H

FIG. 10

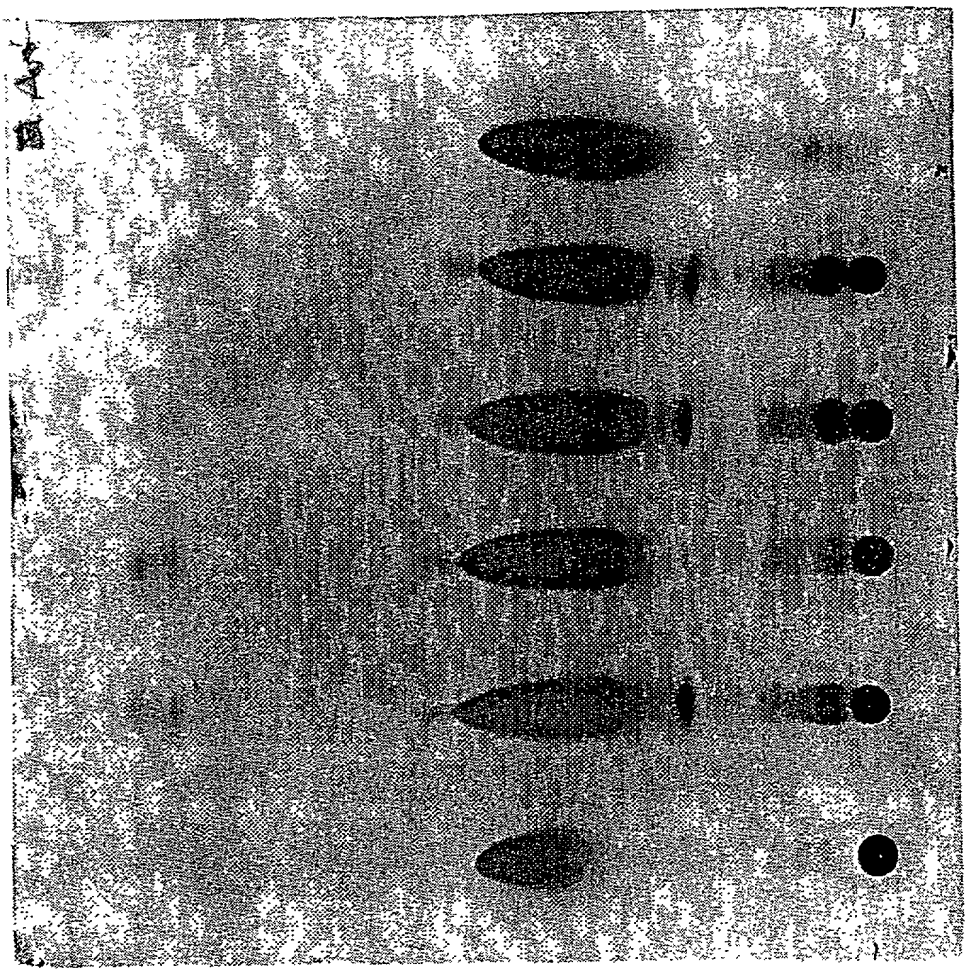


FIG. 11

A B C D E F G H

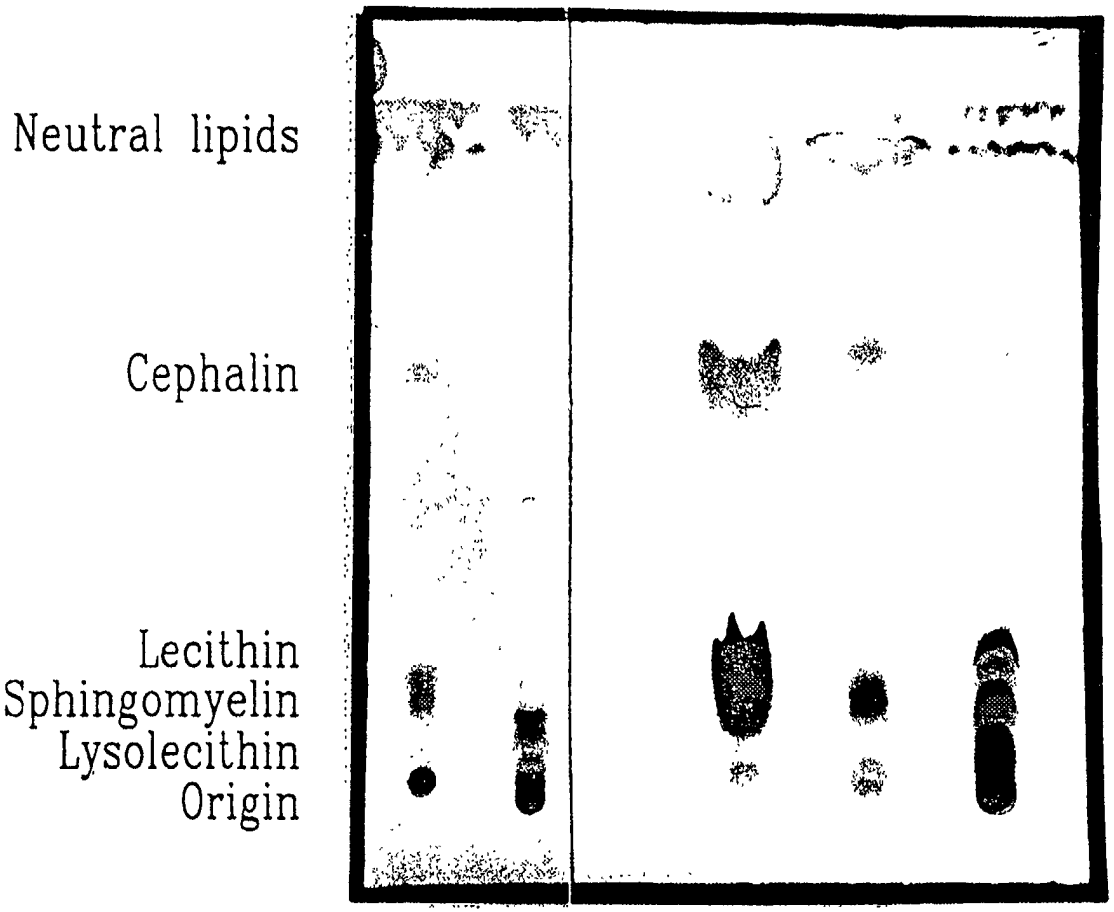


Fig. 12

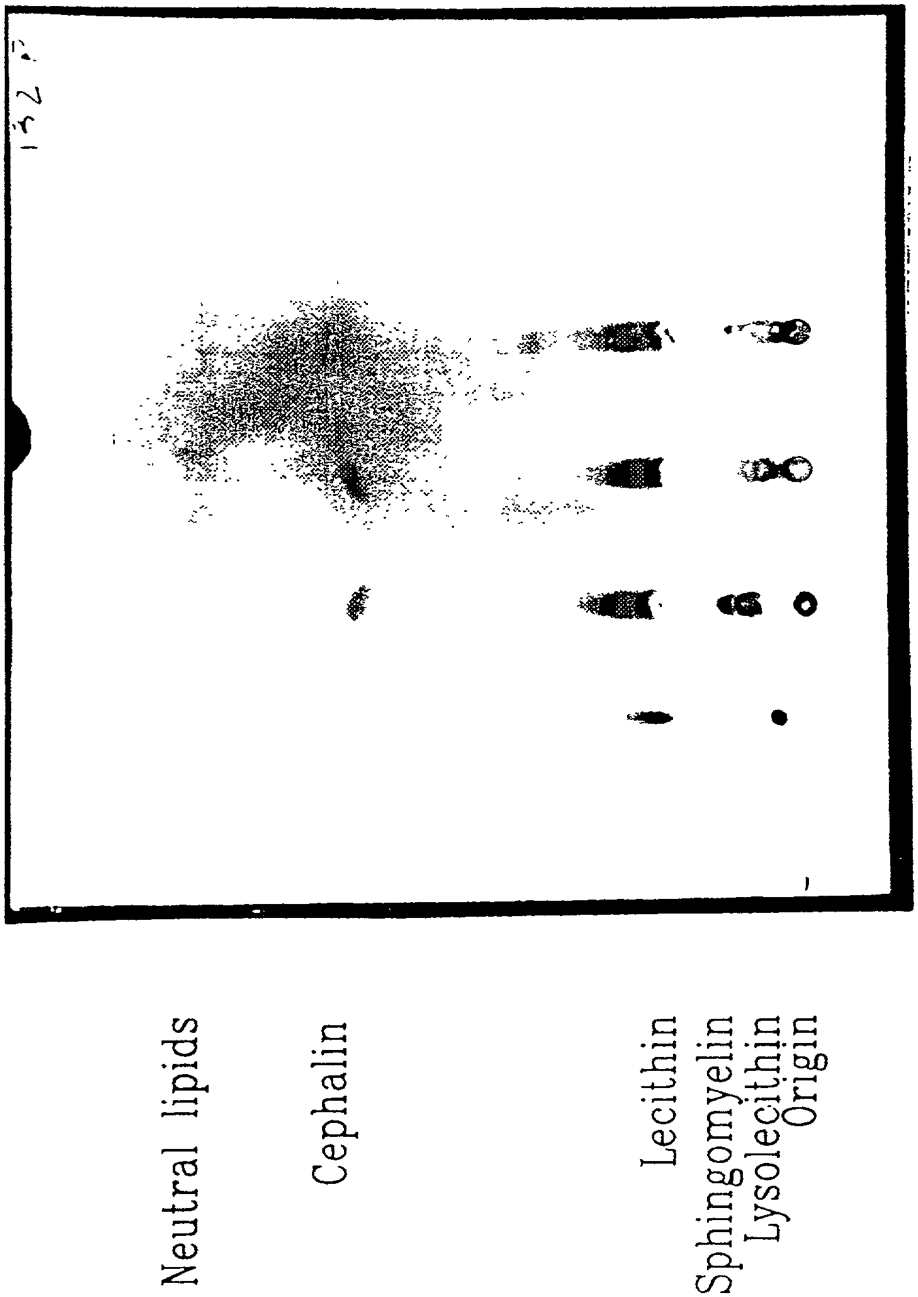


FIG. 13

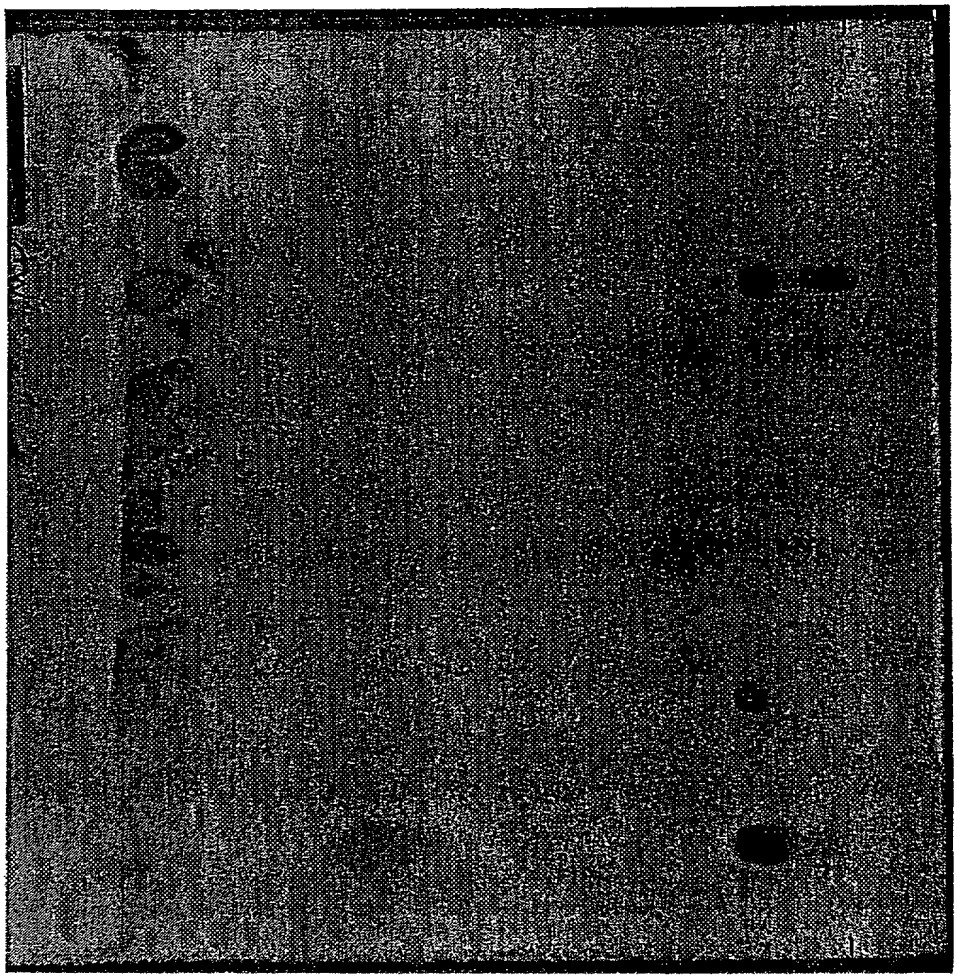


Fig-14

A B C D E F

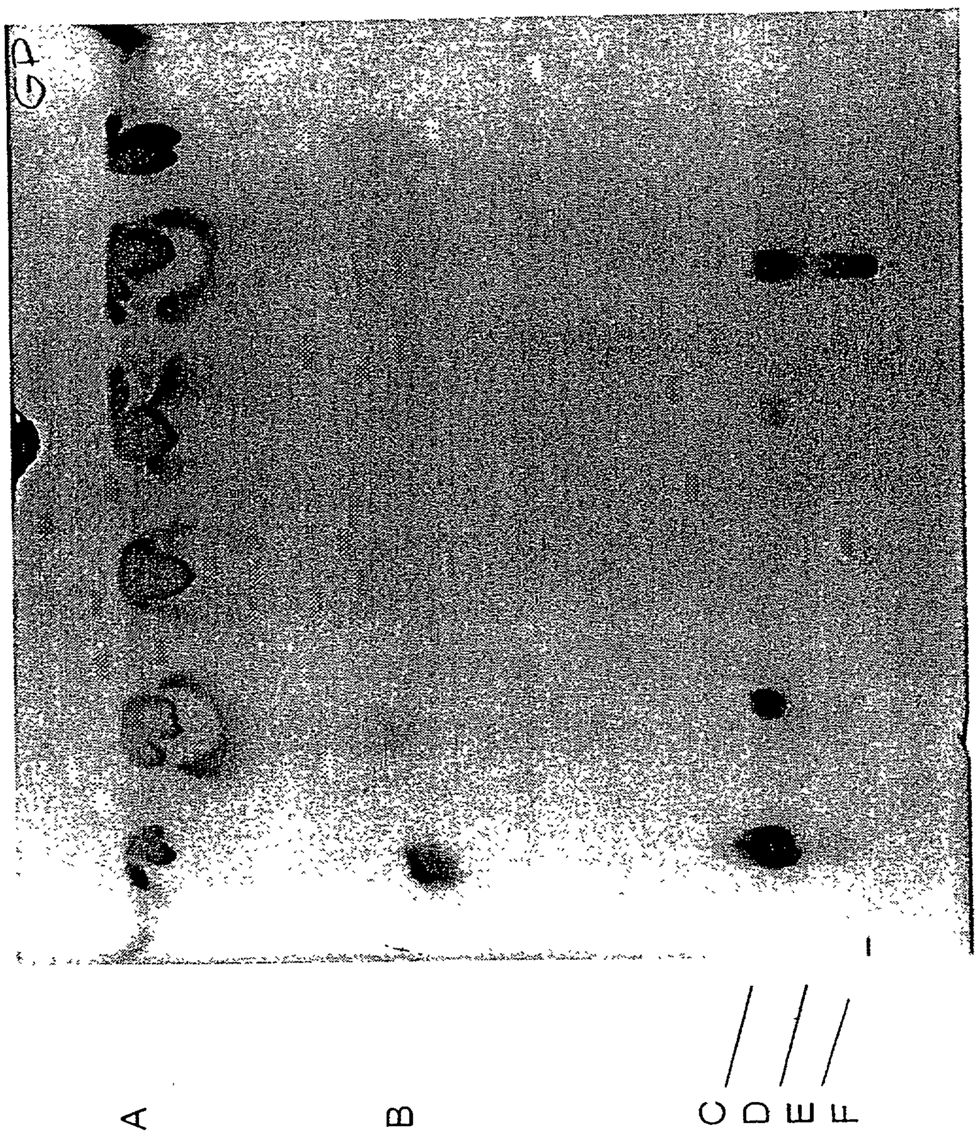


FIG-15

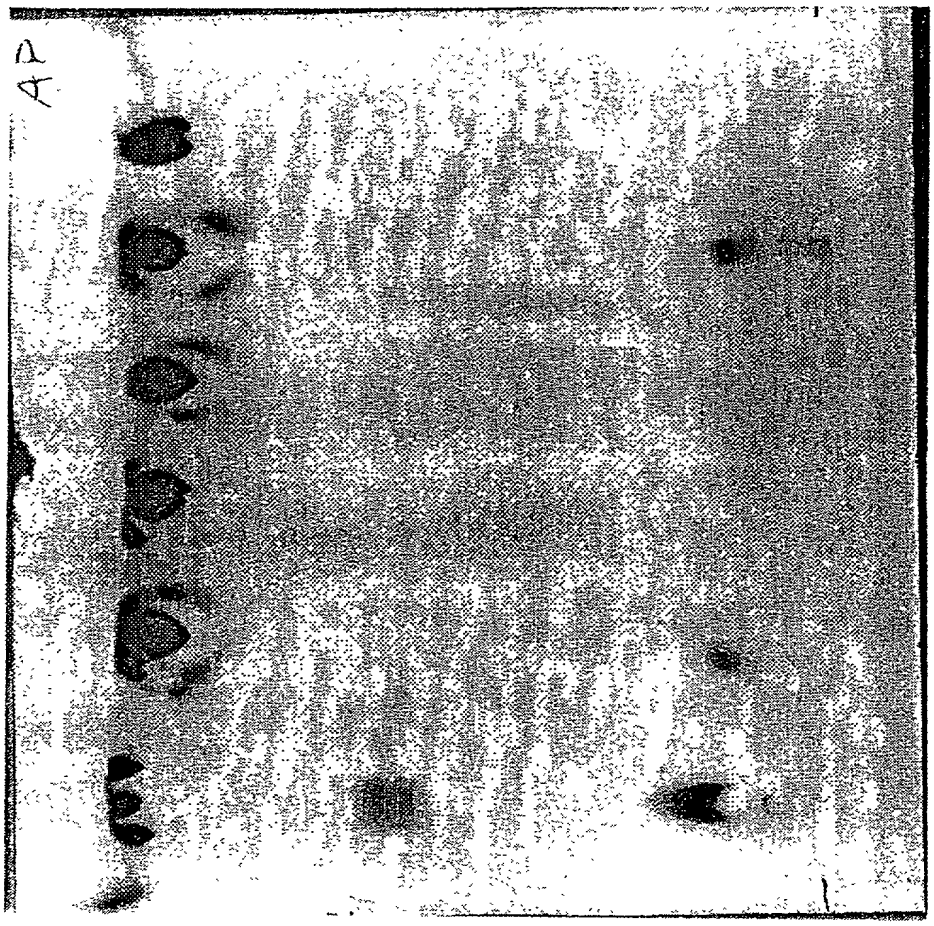


Fig-16

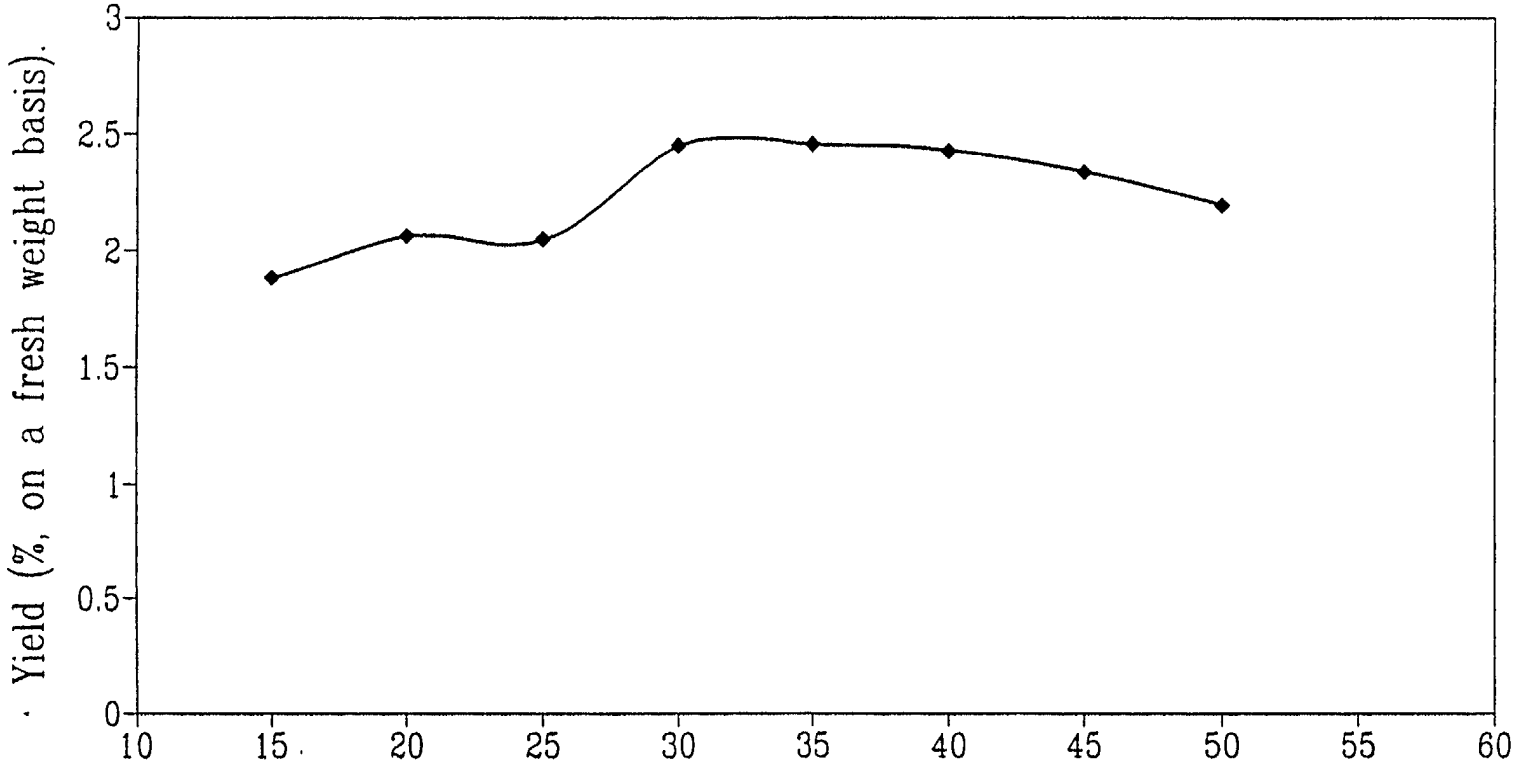
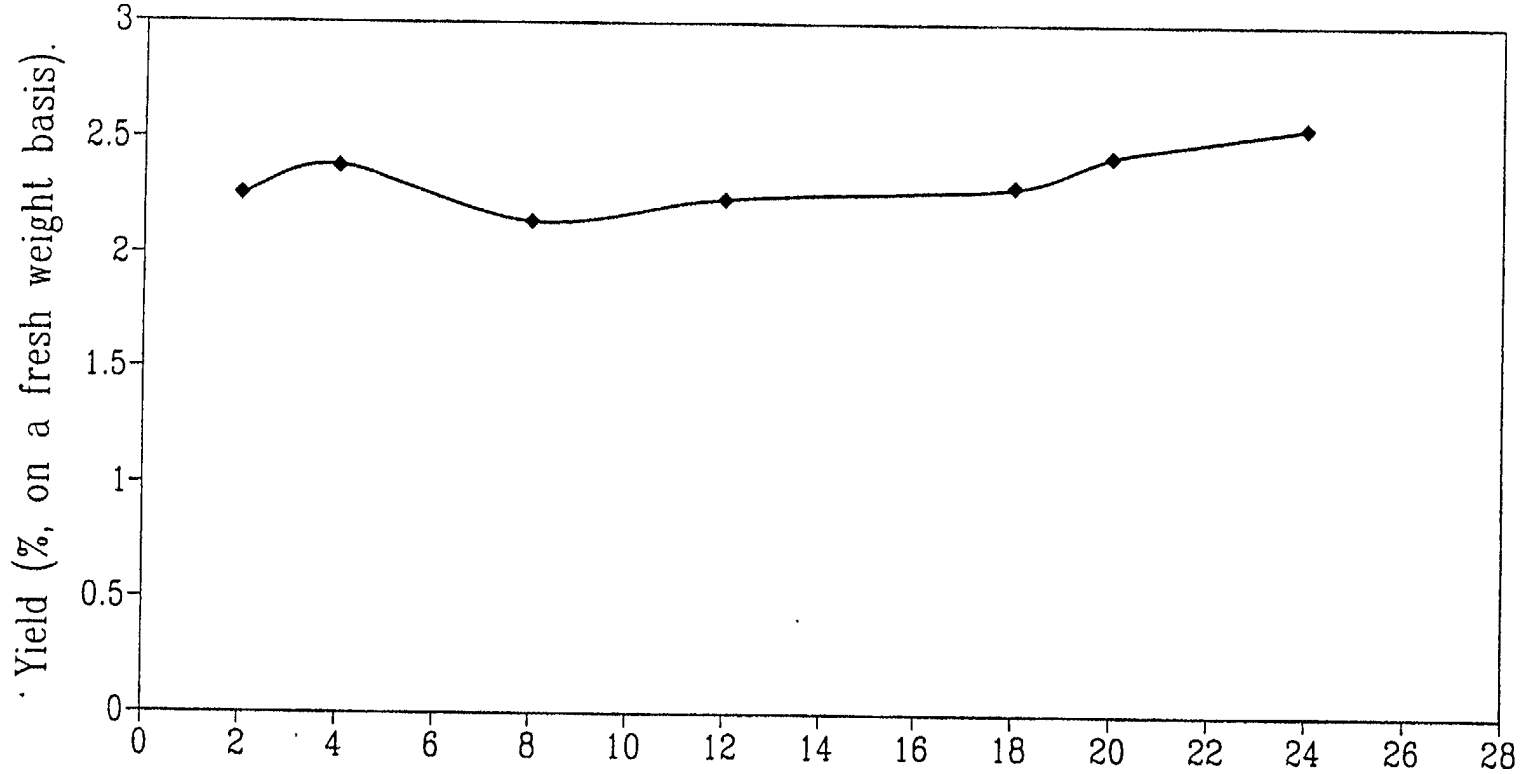


Fig. 17

09/830146



Sample-acetone ratio of 1:9 (w/v).
Determinations in triplicates (variation less than 5 %).

FIG 18

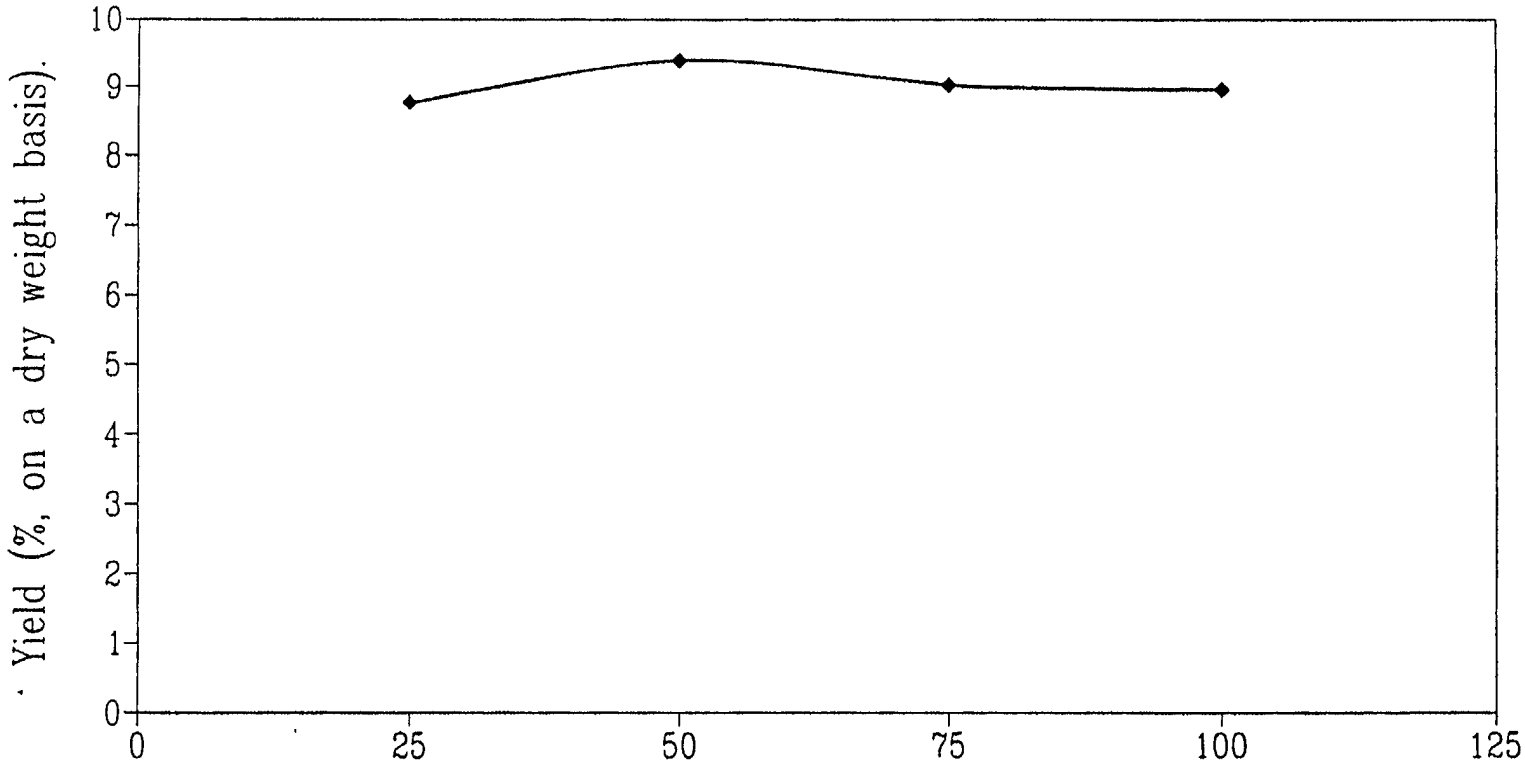

Yield (%, on a dry weight basis).
10
9
8
7
6
5
4
3
2
1
0
0
25
50
75
100
125
Volume of ethanol (mL).
Incubation time of 30 min.
Determinations in triplicates (variation less than 5 %).

Fig. 19

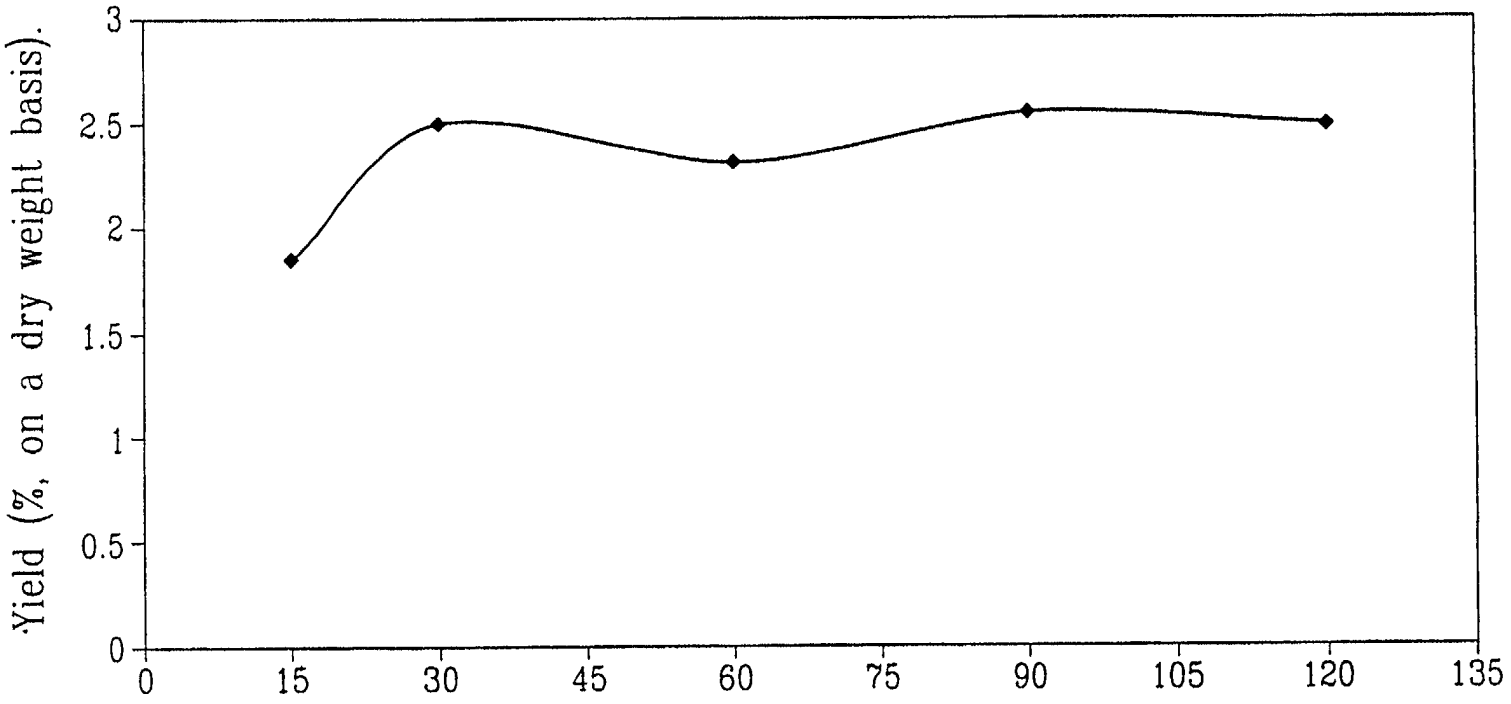

Yield (%, on a dry weight basis).
3
2.5
2
1.5
1
0.5
0
0
15
30
45
60
75
90
105
120
135
Incubation time in ethanol (min).
Sample-ethanol ratio of 1:4 (w/v).
Determinations in triplicates (variation less than 5 %).

FIG. 20

US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER
789-47

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

---

the specification of which (check only one item below):

[] is attached hereto.

[] was filed as U.S. Patent Application Serial Number _
on _,
as amended on _ (if applicable).

[x] was filed as a PCT international application number PCT/CA99/00987 on 21 October 1999 as amended under PCT Article 19 on __ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the applications for which priority is claimed:

PRIOR FOREIGN PATENT APPLICATION(S) AND ANY PRIORITY CLAIMED UNDER 35 U.S.C. §119:

COUNTRY (If PCT Indicate PCT)	APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)	PRIORITY CLAIMED UNDER 35 USC 119
CANADA	2,251,265	21 October 1998	[x] YES [] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO

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COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

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I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NUMBER	U S FILING DATE	PATENTED	ABANDONED	PENDING

PCT APPLICATIONS DESIGNATING THE U.S.

PCT APPLICATION NUMBER	PCT FILING DATE	U S SERIAL NUMBERS			
PCT/CA99/00987	21 October 1999				

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the U.S. Patent and Trademark Office connected therewith: J. Rodman Steele, Jr., Registration No. 25,931; Gregory A. Nelson, Registration No. 30,577, Joseph W. Bain, Registration No. 34,290; Robert J. Sacco, Registration No. 35,667; Mark D. Passler, Registration No. 40,764; Stanley A. Kim, Registration No. 42,730; Steven M. Greenberg, Registration No. 44,725; Neil R. Jetter, Registration No. 46,803; Larry G. Brown, Registration No. 45,834; Kevin T. Cuenot, Registration No. 46,283; Pablo Meles, Registration No. 33,739; Raynaldo K. Whitty, Registration No. 47,176; and Barbara S. Kitchell, Registration No. 33,928.

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Post Office Box 3188
West Palm Beach, FL 33402-3188

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(561) 653-5000

201	FULL NAME OF INVENTOR	FAMILY NAME: BEAUDOIN	FIRST GIVEN NAME: Adrien	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY: Rock Forest CAX	STATE OR COUNTRY: Quebec, Canada	COUNTRY OF CITIZENSHIP: Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 748, boulevard des Vétérans	CITY: Rock Forest	STATE & ZIP CODE/COUNTRY: Quebec, J1N 1Z7, Canada
202	FULL NAME OF INVENTOR	FAMILY NAME: MARTIN	FIRST GIVEN NAME: Geneviève	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY: Sherbrooke	STATE OR COUNTRY: Quebec. Canada	COUNTRY OF CITIZENSHIP: Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 979, McManamy	CITY: Sherbrooke	STATE & ZIP CODE/COUNTRY: Quebec, J1H 2N1, Canada
203	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
A Beaudoin		
DATE: 31 juillet 2001	DATE	DATE

P1011126;1

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(Includes Reference to PCT International Applications)

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the specification of which (check only one item below):

[] is attached hereto.

[] was filed as U.S. Patent Application Serial Number _
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as amended on _ (if applicable).

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			[] YES [] NO
			[] YES [] NO
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P1011126;1

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U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NUMBER	U S. FILING DATE	PATENTED	ABANDONED	PENDING

PCT APPLICATIONS DESIGNATING THE U.S.

PCT APPLICATION NUMBER	PCT FILING DATE	U S. SERIAL NUMBERS			
PCT/CA99/00987	21 October 1999				

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(561) 653-5000

	FULL NAME OF INVENTOR	FAMILY NAME: BEAUDOIN	FIRST GIVEN NAME: Adrien	SECOND GIVEN NAME
201	RESIDENCE & CITIZENSHIP	CITY: Rock Forest	STATE OR COUNTRY: Quebec, Canada	COUNTRY OF CITIZENSHIP: Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 748, boulevard des Vétérans	CITY: Rock Forest	STATE & ZIP CODE/COUNTRY: Quebec, J1N 1Z7, Canada
	FULL NAME OF INVENTOR	FAMILY NAME: MARTIN	FIRST GIVEN NAME: Geneviève	SECOND GIVEN NAME
202	RESIDENCE & CITIZENSHIP	CITY: Sherbrooke CAN	STATE OR COUNTRY: Quebec. Canada	COUNTRY OF CITIZENSHIP: Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 979, McManamy	CITY: Sherbrooke	STATE & ZIP CODE/COUNTRY: Quebec, J1H 2N1, Canada
	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
203	RESIDENCE & CITIZENSHIP	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

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SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
	Geneviève Martin	
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